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Technische Universität München

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by

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Abbreviations

aaRS	aminoacyl-tRNA synthetase
ABA	abscisic acid
ABI1dN	N-terminal deleted version of ABI1 (122-434 aa)
ABI1dN262	N-terminal deleted version of ABI1 (262-434 aa)
ABI1NPA	N-terminal deleted version of ABI1 (122-434 aa) with one mutation Asp177Ala and has no phosphatase activity
ABI1Sac	C-terminal deleted version of ABI1 (1-268 aa)
ABRE	ABA response element
ACC	1-aminocyclopropane-1-carboxylic acid
AD	activation domain
Amp	ampicillin
AP	alkaline phosphatase
APS	ammonium persulfate
AtGluRS	*Arabidopsis* glutamyl tRNA synthetase
AtGluRS(1-110)	N-terminal version of AtGluRS (1-110 aa)
AtGluRS(1-216)	N-terminal version of AtGluRS (1-216 aa)
AtGluRS(1-261)	N-terminal version of AtGluRS (1-261 aa)
AtGluRS(-20-261)	N-terminal version of AtGluRS (1-261 aa) with additional 20 aa appended to the N-terminal
AtGluRS(1-445)	N-terminal version of AtGluRS (1-445 aa)
AtGluRS(217-445)	deleted version of AtGluRS (217-445 aa)
AtGluRS(232-455)	deleted version of AtGluRS (232-455 aa)
AtGluRS(449-719)	deleted version of AtGluRS (449-719 aa)
AtHB	homeodomain protein in *Arabidopsis thaliana*
AtHB6dC119	C-terminal deleted version of AtHB6 (1-119 aa)
AtHB6dC217	C-terminal deleted version of AtHB6 (1-217 aa)
AtHB6dC269	C-terminal deleted version of AtHB6 (1-269 aa)
AtHB7dC	C-terminal deleted version of AtHB7 (1-136 aa) with additional 16 aa appended to the N-terminal
Ath	*Arabidospsis thaliana*
AXR	auxin resistance gene
ATP	adenosine 5'-triphosphate
BAP	benzyladenine
BCIP	5-bromo-4-chloro-3-indolyphosphate
bp	base pair
BSA	bovine serum albumin
CaMV	Cauliflower mosaic virus
cDNA	complementary deoxyribonucleic acid
CE	coupling element
cfu	colony forming units
CIP	calf intestine phosphatase, alkaline

Abbreviations

CTAB	cetyltrimethylammoniumbromid
2,4-D	2,4-Dichlorophenoxyacetic acid
DAPI	4',6'-diamidino-2-phenyl-indol-dihydrochlorid
DAS	days after sowing
DBD	DNA binding domain
DMF	Dimethylformamid
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiotreitol
EDTA	ethylenediamine tetraacetic acid
EMS	methanesulfonic acid ethyl ester
FDA	fluorescein diacetat
GAs	gibberellins
GAS	AtGluRS-antisense line
GluRS	glutamyl tRNA synthetase
GS	AtGluRS-sense line
GST	glutathione S-transferase
GUS	β-D-glucuronidase
GuSCN	Guanidinthiocyanat
HA	hemagglutinin
HD	homeodomain
HRP	horseradish peroxidase
IPTG	isopropyl β-D-thiogalactoside
Kan	kanamycin
kb	kilobase
kDa	kilo Dalton
Lu	light units
LUC	luciferase
MES	2-morpholinoethansulfone acid
MetRS	methionyl-tRNA synthetase
MUG	4-methylumbelliferon-β-D-glucuronid
NBT	nitroblue tetrazolium
NLS	nuclear localization signals (sequences)
NOS	napaline synthase
ONPG	O-nitrophenyl β-D-galactopyranoside
ORF	open reading frame
Ori	origin of replication
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PGRs	plant growth regulators

PMSF	phenylmethylsulfonyl fluoride
Ponceau S	(3-hydroxy-4-(2-sulfo-4-(sulfo-phenylazo) phenylazo)-2,7-naphtalene disulfonic acid)
PP2C	protein serine/threonine phosphatases of type 2C
Rab18	rab-related protein (dehydrin gene family)
Rab18-LUC	luciferase gene under the control of Rab18 promoter
RFU	relative fluorescence units
RLU	relative light units
Rif	rifampicine
RNA	ribonucleic acid
rpm	rotations per minute
RUB	ubiquitin-related protein
SDS	sodium dodecylsulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEMED	N,N,N,N'-tetramethylethylene diamine
Tris	Tris (hydroxymethyl) aminomethane
β-gal	β-galactosidase
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranosid

Amino acids:

A,	Ala	Alanine	M,	Met	Methionine
C,	Cys	Cysteine	N,	Asn	Asparagine
D,	Asp	Aspartic acid	P,	Pro	Proline
E,	Glu	Glutamic acid	Q,	Gln	Glutamine
F,	Phe	Phenylalanine	R,	Arg	Arginine
G,	Gly	Glycine	S,	Ser	Serine
H,	His	Histidine	T,	Thr	Threonine
I,	Ile	Isoleucine	V,	Val	Valine
K,	Lys	Lysine	W,	Trp	Tryptophan
L,	Leu	Leucine	Y,	Tyr	Tyrosine

Bases:

A: Adenine   C: Cytosine   G: Guanine   T: Thymine   U: Uracil

The wild type genes are written in italic capital letters, *e.g. ABI1, ABI2,*
The mutant genes are written in italic lowercase letters, *e.g. abi1, abi2*
The wild type proteins are written in capital letters, *e.g.* ABI1, ABI2
The mutant type proteins are written in lowercase letters, *e.g.* abi1, abi2

Summary

Auxin and abscisic acid (ABA) are two well-known plant hormones. In general, they exhibit antagonistic effects and corporate regulation in some processes during the plant development. To elucidate the molecular mechanism of hormone action, two aspects were pursued in this work, analysis of ABA signal transduction and the screen for hormone mutants.

I. Signal transduction of abscisic acid in *Arabidopsis thaliana*

Abscisic acid (ABA) plays crucial roles in various aspects of plant growth and development, as well as in adaptation to adverse environmental stresses. Substantial progress has been made in the characterization of the ABA signal transduction cascades. The key component of ABA signaling ABI1 regulates several ABA responses. As a target of the PP2C ABI1, the *Arabidopsis thaliana* homeobox protein AtHB6 functions downstream of ABI1 and acts as a negative regulator in ABA signal pathway (Himmelbach *et al.*, 2002). To identify further ABA signaling components, a yeast two hybrid screening with the bait AtHB6 was performed. In this system AtHB7 and AtGluRS (*Arabidopsis thaliana* glutamyl-tRNA synthetase) turned out to be interaction partners of AtHB6. The obtained results support that the AtHB7 and AtGluRS are novel ABA signaling elements linked to or regulating AtHB6 function. Furthermore, AtGluRS was also identified in a yeast two-hybrid screening as an interaction partner of its own, demonstrating its capability to form homodimers.

The interaction of AtHB6 and AtHB7 in yeast was highly dependent on the leucine zipper motif, indicating that this motif is critical for the heterodimerization of AtHB6 and AtHB7. The interaction between AtHB6 and AtHB7 provides evidence for the heterodimerization of homeodomain proteins thereby generating specific transcription factors (Sessa *et al.*, 1993; 1997). These dimers might provide potential integration of information from different signals in the regulation of gene expression.

The homeodomains of HD-ZIP proteins are likely critical for the interaction with AtGluRS. The yeast two-hybrid interaction assay also revealed that AtGluRS interacts not only with AtHB6 and AtHB7 but also with the protein phosphatase 2C ABI1 and ABI2. The interaction of AtGluRS and ABI1 requires the C-terminal region (262-434 amino acids) of ABI1 but does not depend on the functional catalytic domain of ABI1, while the functional catalytic domain of ABI2 seems to play an important role in the interaction with AtGluRS. The *bona fide* interaction of AtGluRS and ABI1 was further confirmed by protein affinity interaction assay *in vitro* as well as by *in vivo* co-immunoprecipitation based on the co-expression of the interacting proteins tagged by

different epitopes in maize protoplasts. The protein cross-interactions between AtHB6, ABI1 (or ABI2) and AtGluRS suggested a ternary protein complex might occur among them. However, the investigation with yeast three-hybrid system revealed no evidence for complex formation among each triangle of AtHB6, AtGluRS and ABI1/ ABI2 or *abi1/ abi2*. On the contrary, the interference of ABI1/ ABI2 as well as *abi1 / abi2* with the protein interaction of AtHB6dC269 and AtGluRS(1-261) were detected even though no two-hybrid interaction was observed between AtHB6dC269 and *abi1* or *abi2*. Hence the competition of AtHB6 and ABI1/ *abi1* in interacting with AtGluRS is proposed. Likewise, the presence of AtGluRS might reduce the interaction of AtHB6 and ABI1.

The function of the interaction between ABI1 and AtHB6 as well as between AtGluRS and AtHB6 was surveyed using a maize protoplast transient expression system. The co-expression of ABI1 and AtHB6 elevated the AtHB6-activated gene expression two fold, while AtGluRS exerted an inhibition in the AtHB6-activated expression of the reporter gene. These results demonstrate a positive regulatory role of ABI1 and a negative effect of AtGluRS on AtHB6 function. A basis for explaining these findings was provided by protein localization studies. Protein localization analysis revealed that the AtGluRS-GUS was strictly localized in the cytoplasm, and the ecotopic expressed AtHB6-GUS was in the nucleus, while the co-expression of both AtGluRS and AtHB6-GUS resulted in both nuclear and cytoplasmic localization of AtHB6-GUS. Thus, AtGluRS is postulated to interact with AtHB6 in the cytoplasm and reduce the nuclear compartmentation of AtHB6. Consequently the function of AtHB6 as the transcriptional regulator is suppressed. The results unravel a new function of AtGluRS as a positive regulator in ABA signal pathway through inhibiting the function of the negative regulator AtHB6.

II. Suppressor screen with the auxin-insensitive mutant *axr1-12* of *Arabidopsis thaliana*

In a screening for secondary mutants of an auxin insensitive mutant *axr1-12*, 42 second site mutants with elevated auxin-sensitivity and recovered ability in silique development were identified.

Preliminary genetic analysis with 8 mutants based on auxin inhibition of root growth showed that 7 of them carry a recessive mutation and one (Nr 214) carries a semi-dominant mutation. Analysis of 5 of these recessive mutants revealed that they all carry a mutation extragenic to AXR1, and among these 5 mutants at least two different suppressor loci were recognized. The recessive mutants exhibit their novel phenotype only when the plants carry the original *axr1-12* mutation*,* indicating that the defect of *axr1-12* is restored by these mutations and acts within the same pathway. Thus, the

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AXR1 might act upstream of the corresponding genes in auxin signal transduction. The semi-dominant mutant Nr 214 was also demonstrated to have a mutation extragenic to AXR1.

Physiological analyses of the mutants demonstrated that all the suppressors restored the phenotype of *axr1-12* in silique development, leaf morphology and auxin sensitivity of root growth. Most of them restored the apical dominance. However, these mutants have different characteristics with respect to root gravitropism, apical hook formation, seed and seed-like structure development. The data suggest that the suppression might function in different steps of auxin signaling transduction.

General Introduction

It is well known that plant growth regulators auxin and abscisic acid (ABA) influence plant development in a variety of ways. In general, auxin promotes cell growth and expansion, cell division, lateral roots formation, apical dominance, as well as development of fruit (Ozga *et al.*, 2002). ABA enhances adaptation to various stresses, and also plays an important role in coordinating normal embryo development in seeds, controlling dormancy and promoting senescence and abscission.

However, these two hormones can exhibit antagonistic effects and corporate regulation in some processes during the plant development. ABA inhibits IAA-induced cell elongation (Pilet, 1989), suppresses the auxin-induced lateral root formation (Suzuki *et al.*, 2001; De Smet *et al.*, 2003) and abolishes the auxin-activated telomerase during the cell cycle (Yang *et al.*, 2002). IAA can reverse the inhibitory effect of ABA on stomatal opening (Irving *et al.*, 1992), present an opposite manner in regulation of turgor pressure (Iino *et al.*, 2001) and ion channel activity (MacRobbie, 1997). Auxin can also affect the localization of the ABA-dependent gene expression in *Arabidopsis* (Suzuki *et al.*, 2001). The findings indicate cross-talk between ABA and auxin signaling. Nevertheless, the molecular mechanism of these two hormones is still far from clear. To elucidate it, this study was introduced.

Part I

Signal transduction of abscisic acid in *Arabidopsis thaliana*

——— **Identification and characterization of protein interaction partners of AtHB6**

1   Introduction

1.1   ABA signal transduction

Abscisic acid (ABA) is a chemical similar to the terminal portion of some carotenoid molecules. The 15 carbon atoms of ABA configure an aliphatic ring with one double bond, two methyl groups, and an unsaturated chain with a terminal carboxyl group (Figure I-1). The scientific origins of ABA were traced to the investigation of inhibitory compounds involved in seed and bud dormancy in the late 1940s. However, it was only isolated and identified in 1960's. When it was identified it was originally called "abscisin II" that promoted the abscission of cotton fruit (Ohkuma *et al.*, 1963), and "dormin" that was purified from sycamore leaves and promotes bud dormancy (Cornforth *et al.*, 1965). The "abscisin II" and "dormin" were found to be identical thus a new name called "abscisic acid" was given because of its supposed involvement in the abscission process (Addicott *et al.*, 1968).
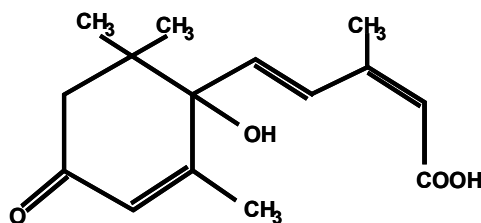
Figure I-1. Abscisic acid (ABA).

In plant ABA has been detected in every organ or living tissues from root cap to the apical bud (Milborrow, 1984). ABA is synthesized almost in all cells containing chloroplasts or amyloplasts. ABA is a weak acid with a pKa 4.7 and its dissociation depends on the pH of each cellular compartment. The protonated form of ABA permeates freely across membranes, but the dissociated anion does not, as a result, the membrane crossing and the distribution of ABA between different compartments depends on their pH values, the alkaline compartment will accumulate more ABA (Cowan *et al.*, 1982). For long distance transport, ABA can be translocated either through the xylem or the phloem (Schurr *et al.*, 1992).

The synthesis of ABA is initiated with the isopentenyl pyrophosphate (IPP) that is the precursor of all terpenoids, in chloroplasts and other plastids. The basic five-carbon units IPP and dimethyallyl pyrophosphate (DMAPP) produce the sesquiterpenes farnesyl pyrophosphate (FPP), subsequently synthesize the C40 xanthophyll zeaxanthin. Zeaxanthin is then converted by a multistep process to 9´-cis-neoxanthin. 9´-cis-neoxanthinis oxidatively cleaved to form the C15 molecule xanthoxin. In the

cytosol the xanthoxin is further converted to ABA-aldehyde and then form ABA through oxidization (Taize and Zeiger, 1998). The identification of some ABA-deficient mutants has been helpful in elucidating the ABA synthesis pathway as well as in understanding the ABA action (Tan *et al.*, 1997; Seo and Koshiba, 2002).

ABA plays crucial roles in various aspects of plant growth and development, as well as in adaptation to adverse environmental stresses (Zeevaart and Creelman, 1988; Leung and Giraudat, 1998). During seed development, ABA is involved in activating the genes encoding seed storage proteins and those encoding a group of proteins designated late-embryogenesis-abundant (LEA) proteins, which are involved in establishing desiccation tolerance (Rock *et al.*, 1995; Ingram and Bartels, 1996). ABA also regulates the induction of seed dormancy and germination, and all these processes are associated with the transcription of special sets of mRNA. During vegetative growth, a major role of ABA is to optimize growth under adverse conditions including water shortage, high osmolarity and low temperature, for instance, upon conditions of water stress, the ABA levels are increased to induce stomatal closure and prevent transpiration thus to limit water loss. This process occurs through rapid changes of ion fluxes in guard cells and osmoregulation (Schroeder *et al.*, 2001). To mediate these developmental and physiological processes, the ABA signaling appears to depend on coordinated interactions between positive and negative regulators including G-proteins, protein phosphatases and protein kinases (Schroeder *et al.*, 2001; Himmelbach *et al.*, 2003).

1.1.1   Identification of ABA signaling intermediates

Significant progress towards understanding ABA action has come from the characterization of mutants that are either defective in ABA biosynthesis or ABA response. To date, nearly 50 genes in *Arabidopsis* have been shown by genetic and/or molecular studies to be involved in ABA biosynthesis or responses and several of them are listed in Table I-1.

Table I-1. Mutants Defective in ABA Synthesis or Response (Finkelstein *et al.*, 2002).

Mutation	Selection/Screen	Phenotype	Gene product	References
aba1	Suppressors of non-germinating GA-deficient lines	ABA deficient; wilty; decreased stress or ABA induction of gene expression; sugar-resistant seedling growth	Zeaxanthin epoxidase	Koornneef *et al.*, 1982; Ishitani *et al.*, 1997; Niyogi *et al.* 1998; Xiong *et al.*, 2001
aba2	Reduced dormancy	ABA deficient; wilty; decreased stress or ABA induction of gene expression; sugar-resistant seedling growth		Leon-Kloosterziel *et al.*, 1996; Laby *et al.*, 2000; Rook *et al.*, 2001

aba3	**Reduced dormancy**	**ABA deficient; wilty; decreased stress or ABA induction of gene expression; freezing sensitive; sugar-resistant seedling growth**	**Aldehyde oxidase Moco**	**Leon-Kloosterziel *et al.*, 1996; Ishitani *et al.*, 1997; Llorente *et al.*, 2000; Rook *et al.*, 2001; Xiong *et al.*, 2001**
aao3	**Wilty phenotype**	**ABA-deficient leaves; wilty, but near-normal seed dormancy**	**Aldehyde oxidase**	**Seo *et al.*, 2000**
abi1-1	**ABA-resistant germination**	**Nondormant seed; pleiotropic defects in vegetative ABA response**	**Protein phosphatase 2C**	**Koornneef *et al.*, 1984; Leung *et al.*, 1994; Meyer *et al.*, 1994**
abi2-1	**ABA-resistant germination**	**Similar to *abi1-1***	**Protein phosphatase 2C**	**Koornneef *et al.*, 1984; Leung *et al.*, 1997; Rodriguez *et al.*, 1998**
abi3	**ABA-resistant germination**	**Pleiotropic defects in seed maturation; vegetative effects on plastid differentiation**	**B3 domain transcription factor**	**Koornneef *et al.*, 1984; Giraudat *et al.*, 1992**
abi4	**ABA-resistant germination**	**Sugar- and salt-resistant germination and seedling growth**	**APETALA2 domain transcription factor**	**Finkelstein, 1994; Finkelstein *et al.*, 1998; Arenas-Huertero *et al.*, 2000; Huijser *et al.*, 2000; Laby *et al.*, 2000; Rook *et al.*, 2001**
abi5	**ABA-resistant germination**	**Slightly sugar-resistant germination and seedling growth**	**bZIP domain transcription factor**	**Finkelstein, 1994; Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000**
gca1, gca2 gca3-gca8	**ABA-resistant root growth**	**Pleiotropic effects on growth, stomatal regulation, and germination**		**Himmelbach *et al.*, 1998; Pei *et al.*, 2000;**
ctr1	**Enhance ABA resistance of *abi1-1***	**Reduced dormancy, constitutive triple response to ethylene**	**Protein kinase (Raf family)**	**Kieber *et al.*, 1993; Beaudoin *et al.*, 2000**
era1	**Enhanced response to ABA at germination**	**Enhanced stomatal response/drought tolerance, meristem defect**	**Farnesyl transferase, β-subunit**	**Cutler *et al.*, 1996**
era3	**Enhanced response to ABA at germination**	**Allelic to *ein2*; ethylene insensitive**	**Membrane-bound metal sensor?**	**Alonso *et al.*, 1999; Ghassemian *et al.*, 2000**
abh1	**ABA-hypersensitive germination and guard cell response**	**Pleiotropic; also enhanced drought tolerance**	**mRNA CAP binding protein**	**Hugouvieux *et al.*, 2001**
sad1	**Supersensitive to ABA and drought induction of RD29::LUC**	**Reduced ABA biosynthesis, hypersensitive to ABA inhibition of germination and root growth**	**U6-related Sm-like small ribonucleo protein**	**Xiong *et al.*, 2001**
hyl1	**Hyponastic leaves**	**Hypersensitive to ABA**	**Double-stranded RNA binding protein**	**Lu and Fedoroff, 2000**

The mutants *aba1*, *aba2*, *aba3*, and *aao3* block the chain of ABA biosynthesis at different steps. These mutants exhibit a wilty phenotype and decreased expression of genes that can be induced by stress or ABA. Their phenotypes can be reversed to wild type by exogenous

application of ABA (Koornneef *et al.*, 1982; Marin *et al.*, 1996; Schwartz *et al.*, 1997; Seo *et al.*, 2000). The characterization of these mutants, together with physicochemical studies, has enabled the elucidation of the pathway of ABA biosynthesis in higher plants.

Moreover, ABA-insensitive (*abi1-abi8, gca1-gca8*, *etc*.) mutants and enhanced response to ABA (*era1-era3, ctr1, abh1, sad1* and *hyl1*, *etc*.) mutants have alterations in their response to ABA. These mutants lead to the identification of likely components of ABA signal transduction cascades.

The ABA insensitive mutants *abi1* to *abi8* were identified by selecting for seeds capable of germinating in the presence of ABA concentration that is inhibitory to the wild type (Finkelstein, 1994; Koornneef *et al.*, 1984; Finkelstein and Lynch, 1997). These mutants exhibit additional defects. The *abi1* and *abi2* mutants have non-dormant seeds and pleiotropic defects in vegetative ABA response, and *abi3* exhibits pleiotropic defects in seed maturation (Koornneef *et al.*, 1984). These three genes thus seem to mediate the inhibitory effects of endogenous ABA on seed germination. The *ABI1* and *ABI2* gene were found to encode homologous protein phosphotases and were suggested to function as central regulators in the ABA signal relay (Leung *et al.*, 1994, 1997; Meyer *et al.*, 1994; Rodriguez *et al.*, 1998). ABI3, ABI4 and ABI5 are three transcription factors linked to ABA response. The *ABI3* gene is the ortholog of maize *VP1*; it encodes a protein that functions as a transcription factor essential for ABA action in seeds (Giraudat *et al.*, 1992). *abi4* exhibits resistance to sugar and salt stress during germination and seedling growth, and decreased sensitivity to ABA inhibition of germination. The protein ABI4 contains an APETALA2-like DNA binding domain (Finkelstein *et al.*, 1998). ABI5 is a member of a basic leucine zipper transcription factor family (Finkelstein and Lynch, 2000).

Another series of ABA insensitive mutants, *gca1 - gca8*, were isolated based on their reduced sensitivities to the inhibition of seedling root growth by exogenouse ABA. They also exhibit pleiotropic effects on growth, stomatal regulation, and germination. The *gca1* and *gca2* mutants display a disturbed stomatal regulation similar to the *abi1* and *abi2* mutants, however, both ABA and ethylene affect the growth of all the other *gca* mutants (Benning *et al.*, 1996; Himmelbach *et al.*, 1998), implying cross-talk between the functional pathways of ABA and ethylene.

The enhanced response to ABA mutants of *Arabidopsis*, *era1* and *era3*, were identified by reduced seed germination in the presence of low concentrations of ABA that are not inhibitory to the wild type (Cutler *et al.*, 1996). In addition, the *era1* mutant exhibits enhanced seed dormancy, stomatal response and drought tolerance (Pei *et al.*, 1998). The *ERA1* gene encodes the β subunit of a farnesyl transferase, which may possibly function as a negative regulator of ABA signaling by modifying signal transduction proteins for

membrane localization (Cutler *et al.*, 1996). ERA1 is expressed in guard cells. Double-mutant analyses of *era1* with the ABA-insensitive mutants *abi1* and *abi2* showed that *era1* suppresses the ABA-insensitive phenotypes. Moreover, *era1* plants exhibited a reduction in transpirational water loss during drought treatment (Pei *et al.*, 1998). The mutant *era3* is an allelic of *ein2*; it exhibits ethylene insensitivity. It was suggested that the *ERA3* gene encodes a membrane-bound metal sensor (Alonso *et al.*, 1999; Ghassemian *et al.*, 2000). Another mutant with enhanced response to ABA is *ctr1*. It enhanced the abscisic acid (ABA)-resistant seed germination phenotype of the *Arabidopsis abi1-1* mutant, and the *CTR1* gene encodes a protein kinase of the Raf family involved in ethylene signaling (Kieber *et al.*, 1993; Beaudoin *et al.*, 2000).

abh1, sad1 and hyl1 belong to another kind of ABA-hypersensitive mutants, their corresponding genes encode RNA binding proteins. The *Arabidopsis abh1* mutant shows ABA-hypersensitive stomatal closing and seed germination, *ABH1* encodes the *Arabidopsis* homolog of a nuclear mRNA cap binding protein. Interestingly, the *abh1* mutant was shown to enhance the ABA-induced $[Ca^{2+}]_{cyt}$ elevation in guard cells, suggesting that ABH1 is a modulator of ABA signaling. It is proposed to function by transcript alteration of early ABA signaling elements (Hugouvieux *et al.*, 2001). The *Arabidopsis sad1* mutant is ABA-hypersensitive during seed germination and root growth, as well as in expression of stress-response genes. The *SAD1* gene encodes a protein with homology to one of the Sm-like small nuclear ribonucleoproteins (snRNAs) that participate in mRNA splicing, export, and degradation (Xiong *et al.*, 2001). The *hyl1* mutant is hypersensitive to ABA in seed germination and root growth, but shows reduced sensitivity to auxin and cytokinin. Transcripts of a number of stress-response genes, including glutathione-S-transferase, *PR1* genes and the *AtMPK3* gene, *etc.*, are substantially more abundant in the mutant than in the wild type plants. The HYL1 protein is a nuclear double-stranded RNA (dsRNA) binding protein (Lu and Fedoroff, 2000). Study of these RNA binding proteins could lead to the elucidation of new posttranscriptional regulatory mechanisms.

1.1.2 Protein phosphorylation /dephosphorylation events in ABA signaling

Nearly all biological signaling systems involve protein phosphorylation reactions at some step in the pathway. In ABA signaling, several protein kinases and phosphatases are involved in and act coordinately as the positive or negative regulators (Finkelstein *et al.*, 2002; Himmelbach *et al.*, 2003).

Protein kinases

Broad-range protein kinase antagonists such as K-252a inhibited ABA-induced stomatal

closing in multiple species (Allen *et al.*, 1999; Hey *et al.*, 1997; Schmidt *et al.*, 1995). K252a also inhibited the ABA-activation of various target genes in pea epidermal peels (Hey *et al.*, 1997) and tomato hypocotyls (Wu *et al.*, 1997). These results underline the importance of protein kinases as positive regulators of ABA action. Moreover, progress has been made recently in the identification of protein kinases involved in ABA action. An ABA activated serine-threonine protein kinase, designated AAPK, was isolated from guard cells; the transient transformation of guard cells with a dominant mutant form AAPK impairs ABA activation of S-type anion channels and disrupts ABA-induced stomatal closing, indicating AAPK is essential for ABA-induced stomatal closure (Li *et al.*, 2000). The protein kinase PKABA1 can be induced by ABA and suppresses GA-inducible gene expression in barley aleurone layers (Gomez-Gadenas *et al.*, 1999). The CDPKs, which directly bind calcium and have calcium-stimulated kinase activities, also mediate ABA signaling. The expression of constitutively active AtCPK10 and AtCPK30 leads to activation of the ABA-inducible barley HVA1 promoter (Sheen, 1996). A CDPK was also reported to phosphorylate the KAT1 potassium channel, which might be required for stomatal closure (Li *et al.*, 1998). It was supposed that CDPK may function upstream of ABA-activated Ca^{2+}-independent kinases in *Vicia* guard cells (Mori and Muto, 1997). ABA activation of additional types of protein kinases has been shown in barley aleurone protoplasts (Knetsch *et al.*, 1996) and in pea epidermal peels (Burnett *et al.*, 2000). However, the application of protein kinases inhibitors was able to restore ABA responses in the guard cells of ABA-insensitive *abi1-1* transgenic (Armstrong *et al.*, 1995) or mutant (Pei *et al.*, 1997) plants, indicating that protein kinases sometimes also block ABA signaling.

Protein phosphatases

ABA signaling is also regulated by diverse serine/threonine protein phosphatases, especially PP2C. Genetic evidence has implicated three *Arabidopsis* PP2C (AthPP2CA, ABI1, and ABI2) as negative regulators of ABA signal pathway (Gosti *et al.*, 1999; Merlot *et al.*, 2001; Tahtiharju and Palva, 2001). Their amino acid sequences are highly identical (Figure1).

Expression of AtPP2CA was induced by low temperature, drought, high salt and ABA. Transgenic antisense AtPP2CA plants displayed increased sensitivity to ABA, and furthermore, the expression of cold- and ABA-induced genes was enhanced in these plants. The results suggest that AthPP2CA is a negative regulator of ABA responses during cold acclimation (Tahtiharju and Palva, 2001). AtPP2CA regulates the activity of the K^+ channel AKT2 (Cherel *et al.*, 2002); it also interacts with the AKT3 potassium channel protein (Vranova *et al.*, 2001).

ABI1 and ABI2 proteins belong to Mg^{2+}-dependent serine/threonine protein phosphatases (PP2Cs) and regulate ABA signaling both in seeds and vegetative tissues (Himmelbach *et al.*, 1998; Leung and Giraudat, 1998). ABI1 and ABI2 display a similar architecture and their C-terminal domains that contain the PP2C core share 86% amino acid identity, while their N-terminal extensions are less conserved (49% identity) (Leung *et al.*, 1994; 1997; Meyer *et al.*, 1994). The N-terminal extension of ABI1 contains a sequence motif that matches a type of calcium-binding domain known as the EF hand, but there is no evidence supporting that Ca^{2+} regulates ABI1. The *Arabidopsis* mutants *abi1* and *abi2* carry an identical mutation (Gly to Asp, position 180 in abi1, position 168 in abi2) within the catalytic domain, this leads to the deficiency in catalytic activity of abi1 and abi2 and reduction of the plant's responsiveness to ABA (Leung *et al.*, 1997; Leube *et al.*, 1998; Rodriguez *et al*, 1998). These two mutant proteins mediate a dominant ABA-insensitive phenotype. However, the allelic intragenic revertants of *abi1* and *abi*2 were isolated and showed a recessive ABA-sensitive phenotype (Gosti *et al.*, 1999; Merlot *et al.*, 2001). The *in vitro* enzymatic assay of the proteins encoded by corresponding revertants showed an apparent reduction of PP2C activity. The transient expression studies with over-expression of wild-type ABI1 in maize mesophyll protoplasts revealed that the ABA signaling was blocked (Sheen, 1998). These results indicate that the ABI1 and ABI2 are negative regulators of the ABA signal pathway.

A new PP2C with homology to ABI1 and ABI2 has been characterized (Figure I-2), it is named AtP2C-HA (for homology to ABI1/ABI2). The amino acid sequence of the C-terminal PP2C catalytic domain of AtP2C-HA is 64% and 62% identical to ABI1/ABI2, respectively. Its expression is notably up-regulated in response to ABA (Rodriguez, 1998), they are probably the negative regulators of ABA signaling like ABI1 and ABI2.

	ABI1	ABI2	AtP2C-HA	AthPP2CA
ABI1 (434 aa)	100	80	56	45
ABI2 (423 aa)		100	54	44
AtP2C-HA (511 aa)			100	41
AthPP2CA (399 aa)				100
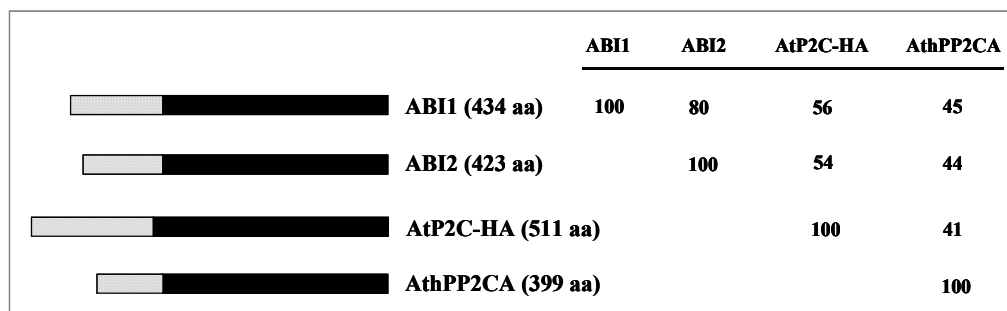
Figure I-2. Schematic diagram of plant PP2C (left) and percentage of amino acid sequence identity between any pair of proteins (right). The N-terminal extensions and C-terminal regions representing the protein phosphatase catalytic domain are indicated by hatched boxes and solid boxes, respectively.

In eukaryotes, PP2C has been implicated as a negative regulator of protein kinase cascades that are activated as a result of stress, for instance, human PP2C alpha dephosphorylated and inactivated MAPKKs in the stress-responsive MAPK cascades (Takekawa *et al.*, 1998). In fission yeast, genetic studies suggest that PP2Cs are involved in the negative regulation of the stress signal that is transmitted through the Wis1-Spc1 MAPK cascade (Shiozaki and Russell, 1995), and a plant protein phosphatase 2C, MP2C, acts as a negative regulator of a wound-activated MAPK pathway (Meskiene *et al.*, 1998). PP2Cs are assumed to play important role in regulating the protein kinases in some way and further regulating gene transcription or ion channels in the ABA signal relay.

ABI1 and ABI2 were also suggested to be involved in the modulation of the ion channel and secondary messengers that were regulated by ABA. The *abi1* mutation eliminates ABA activation of both outward- and inward-rectifying K^+ channels in the plasma membrane (Armstrong *et al.*, 1995). The *abi1* and *abi2* mutations also interfere with ABA activation of slow anion channels (Pei *et al.*, 1997; Allen *et al.*, 1999). The response of anion channels to ABA was restored by the kinase inhibitor K-252a in the *abi1* mutant, but not in the *abi2* mutant, although the kinase inhibitor alone did not activate the response (Pei *et al.*, 1997). The *abi1* and *abi2* mutations cause a reduction in the ABA-induced increases in cytoplasmic calcium in guard cells but do not interfere with Ca^{2+}-induced stomatal closure (Allen *et al.*, 1999). These data demonstrate that the dominant *abi* PP2C mutants interfere with very early ABA signaling events that act upstream of $[Ca^{2+}]_{cyt}$ (Allen *et al.*,1999, Figure2). Recent studies raise the possibility that redox regulation and the production of reactive oxygen species (ROS) are centrally involved in ABA signaling through their influence on Ca^{2+} channels. Micromolar concentration of ABA stimulate the generation of H_2O_2 in guard cells, H_2O_2 triggers stomatal closing, and the stomatal closing is inhibited when H_2O_2 production is blocked by inhibiting the NADPH oxidase with diphenylene iodonium chloride (Pei *et al.*, 2000). H_2O_2 activates Ca^{2+} currents in *abi1*, but not in the *abi2* mutant. The protein phosphatases ABI1 and ABI2 are rapidly inactivated by H_2O_2 (Leube *et al.*, 1998; Meinhard and Grill, 2001; Meinhard *et al.*, 2002) and represent important targets for redox-regulation of the ABA pathway.

Phosphorylation events in the ABA-induced stomatal closing

The ABA induced-stomatal closing has been a relatively integrated system for studying ABA signal transdution. The stomatal closing is mediated by a reduction in the turgor pressure of guard cells, which is generated by the efflux of K+ and anions, sucrose removal and the conversion of malate to osmotically inactive starch. It was evidenced that phosphorylation events are central regulation steps during these processes. The

Ca^{2+}-independent protein kinases AAPK was proved in the transient transformed guard cells to positively regulate the ABA activation of S-type anion channels and the ABA-induced stomatal closing (Li *et al.*, 2000). Two Ca^{2+}-dependent protein kinases CDPKs was shown to act upstream of the AAPK (Mori and Muto, 1997) and overexpression studies on maize protoplasts further indicate that CDPKs may be positive regulators in ABA signal transduction (Sheen, 1996). MAP kinases have also been reported to positively control the ABA-induced stomatal closure in *Pisum* (Burnett *et al.*, 2000). Inhibitors of PP1 and PP2A protein phosphatases OA (okadaic acid) could enhance S-type anion and ABA-induced stomatal closure (Schmidt *et al.*, 1995; Grabov *et al.*, 1997; Hey *et al.*, 1997), suggesting that PP1s and PP2As act as negative regulators in ABA signaling. However, in *Arabidopsis*, OA partially inhibited ABA activation of S-type anion channels and stomatal closing (Pei *et al.*, 1997), suggesting that some PP1s and PP2As can also act as positive regulators in ABA signaling. Figure3 presents a model for the proposed functions of positive and negative regulators in the ABA-induced stomatal closing (Schroeder *et al.*, 2001).
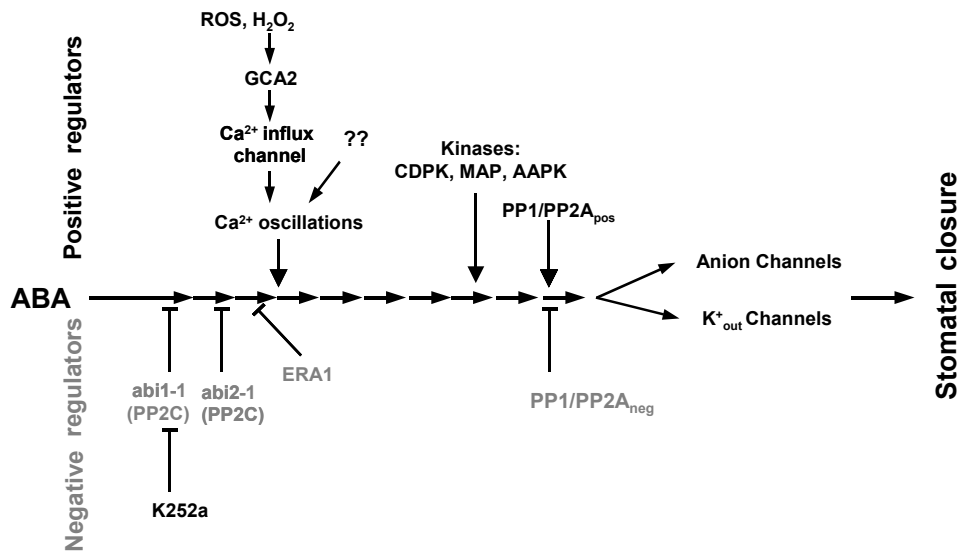
Figure I-3. ABA signal transduction in guard cells. A working model for the proposed functions of positive (top) and negative regulators (bottom) of ABA-induced stomatal closing. (According to Schreoder *et al.*, 2001).

1.1.3   ABA-regulated genes

Many of the actions of ABA, in both seeds and vegetative tissues, involve

modification of gene expression at the transcriptional level. Some of those genes are ABA-inducible or -repressible. Recently, cDNA microarray analysis showed that the transcripts of 245 genes increased after ABA treatment. Among these ABA-inducible genes, 22 transcription factor genes were identified (Seki *et al.*, 2002). Applying the massively parallel signature sequencing (MPSS) to study genome-wide ABA-responsive gene expression, 1354 genes that are either up- or down-regulated following ABA treatment of *Arabidopsis* WT seedlings were identified as well (Hoth *et al.*, 2002). The isolation of genes responsive to ABA, coupled with promoter analyses, have provided insights into the biological function of the encoded proteins, as well as into the nature of the *cis*- and *trans*-acting factors involved in ABA responsiveness (Busk and Pages, 1998).

To date, several groups of *cis*-acting elements that are involved in ABA responsiveness have been identified. One main group is the ACGT-containing G-box elements designated ABA response elements (ABREs); the element defined as a sequence of 8-10 base pairs with the ACGT core (*e.g.* (C/T)ACGTGGC) is required for responsiveness to ABA. The ABA-inducible genes *Em* from wheat (Marcotte *et al.*, 1989; Guiltinan *et al.*, 1990), *rab16A* and *OsEm* from rice (Mundy *et al.*, 1990; Hattori *et al.*, 2002), *rab17* from maize (Busk *et al.*, 1997), barley gene *HVA1* (Straub *et al.*, 1994), and the carrot gene *Dc3* (Siddiqui *et al.*, 1998), were shown to contain this kind of element. A well characterized ABA-inducible and drought-inducible promoter from carrot *LEA Dc3* gene has been used to visualize gene expression induced by ABA and drought after fusion to the *GUS* gene and expression in wild-type, *aba1, abi1* and *abi2* mutant (Siddiqui *et al.*, 1998; Chak *et al.*, 2000). The ABRE elements can be recognized by bZIP transcription factors (Marcotte *et al.*, 1989; Mundy *et al.*, 1990). There are *cis*-elements designated "coupling elements" which are active in combination with ABRE but not alone. The coupling elements CE1 and CE3 in the barley genes *HVA22* and *HVA1* are necessary for activation by ABA (Shen and Ho, 1997). Other *cis*-acting elements involved in ABA responsiveness are RY/Sph elements. The maize *C1* gene contains an Sph element (Suzuki *et al.*, 1997). The RY/Sph elements can be bound by B3 domain proteins. Sequences recognized by MYB- and MYC-class transcription factors are the third group of ABA-response *cis*-acting elements (Abe *et al.*, 1997). A 67-bp DNA fragment of the promoter of the dehydration-responsive gene, *rd22*, was shown to be sufficient for dehydration- and ABA-induced gene expression, and the putative recognition sites for the basic helix-loop-helix protein MYC and for MYB in this fragment were shown to function as *cis*-acting elements in the dehydration-induced expression of the *rd22* gene in transgenic tobacco (Abe *et al.*, 1997; Abe *et al.*, 2003). Recently, a pseudopalindromic core sequence in the AtHB6 promoter was reported to be recognized by AtHB6 itself and was involved in gene expression induced by ABA

(Himmelbach *et al.*, 2002).

Corresponding to the *cis*-acting elements, several transcription factors, EmBP-1, VP1, ABI3, ABI4, ABI5, AREBs and GF14 (the 14-3-3 protein), have been proposed to be involved in ABA-inducible transcription.

The ABRE elements can be recognized by bZIP transcription factors. One bZIP subfamily in *Arabidopsis* has been linked genetically to ABA response. It is composed of ABI5 and its homologs, the ABRE binding factors (ABFs and AREBs) (Choi *et al.*, 2000; Uno *et al.*, 2000). They are correlated with ABA-, seed- or stress-induced gene expression in sunflower and rice (Kim *et al.*, 1997; Kim and Thomas, 1998; Hobo *et al.*, 1999). *ABI5* was identified independently by homology with a sunflower gene isolated *via* a yeast one-hybrid screen using the ABA-responsive *Dc3* promoter as "bait". ABI5 contains conserved a bZIP region which is thought to be involved in DNA binding and potential protein dimerization; it also contains six predicted serine/ threonine phosphorylation sites. ABI5 is important for regulation of some *LEA* genes (like *AtEm1*, *AtEm6*) from late embryonic growth through early seedling development (Finkelstein and Lynch, 2000; Brodcard *et al.*, 2002). A rice homolog of ABI5, TRAB1, identified by a two-hybrid screen using the basic domains of OsVP1 as bait, was shown to interact with ABREs *in vitro* and to activate ABA-inducible transcription in rice protoplasts (Hobo *et al.*, 1999). Additional ABRE binding factors, the AREB1 and AREB2, were identified as the ABRE-binding proteins of *rd29B* by using one-hybrid system. The transcription of the *AREB1* and *AREB2* genes is up-regulated by drought, NaCl, and ABA treatment in vegetative tissues. In protoplast transient expression, the transcriptional activation of the ABRE-driven reporter by AREB1 and AREB2 requires ABA (Uno *et al.*, 2000).

Arabidopsis ABI4 shows high homology to the transcriptional regulators APETALA2(AP2) domain proteins, which are related closely to the drought response element binding (DREB)/CBF subfamily of the domain family (Finkelstein *et al.*, 1998). A seed-specific maize ABI4 was shown to bind to the CE1 element in a number of ABA-related genes (Niu *et al.*, 2002).

Another ABI transcriptional factor is ABI3. ABI3 in *Arabidopsis* and VP1 in maize are orthologous genes encoding transcription factors of the B3 domain family. They encode seed-specific, homologous transcription factors that mediate ABA-regulated gene expression in seeds and play key roles in ABA-dependent seed maturation (McCarty *et al.*, 1991; Giraudat *et al.*, 1992). The *Arabidopsis*, maize, rice, bean, and resurrection plant VP1 orthologs all have been shown to activate the transcription of ABA-inducible promoters *in vivo*. Furthermore, the conserved B3 domain of VP1 binds *in vitro* to the conserved RY element present in many seed-specific promoters, including those of the

C1 and *Em* genes (Suzuki *et al.*, 1997). VP1 enhances transcription of *C1* gene through a sequence including the Sph element (Suzuki *et al.*, 1997).

In *Arabidopsis*, the MYC and MYB proteins, which were suggested to be involved in expression of the *rd22* gene (Abe *et al.*, 1997), are expressed in response to abiotic stress and drought or ABA, and the MYB/ MYC response system might regulate slow adaptive responses to dehydration stress (Shinozaki and Yamaguchi-Shinozaki, 2000). Further study showed that AtMYC2 and AtMYB2 interact specifically with the MYC and MYB recognition sites of rd22 promoter. Several ABA-induced genes including rd22 and AtADH1 were upregulated in the transgenic plants with over-expression of AtMYC2 and/or AtMYB2. Thus both AtMYC2 and AtMYB2 proteins function as transcriptional activators in ABA-inducible gene expression (Abel *at el.*, 2003).

The expression of some homeodomain leucine-zipper proteins (AtHB6, AtHB7, AtHB12) can be induced by ABA or abiotic stress. A recent report showed that the *Arabidopsis* homeodomain protein AtHB6 recognizes a *cis*-element present in its own promoter and mediates AtHB6 and ABA-dependent gene expression in protoplasts (Himmelbach *et al.*, 2002). It is possible that most of these homeodomain leucine zipper proteins have roles in ABA response.

Most of the transcriptional factors have the potential to be phosphorylated and dephosphorylated, for example, ABI5 contains six predicted serine/ threonine phosphorylation sites (Finkelstein and Lynch, 2000) and ABI4 contains a serine/threonine-rich domain (Finkelstein *et al*., 1998). These sites could be the regulated sites of serine-threonine protein kinases, *e.g.* the Pto-like kinases in plants related to resistance (Di Gaspero and Cipriani, 2003), or serine/threonine phosphatases such as the well known ABI1 and ABI2. It is possible that the function of these transcriptional factors is regulated by protein phosphorylation/ dephosphorylation events.

1.2 Homeodomain protein

Homeobox genes were first isolated from *Drosophilia melanogaster*, and then from other animals and plants. *Drosophilia* homeobox genes have been demonstrated to be important in homeotic transformations during embryogenesis (McGinnis *et al.*, 1984; Scott and Weiner, 1984). The first homeobox gene identified from plants was *KNOTTED-1* in maize (Vollbrecht *et al.*, 1991) and was shown to play an important role in shoot apical meristem maintenance during plant development (Smith *et al.*, 1992).

1.2.1   Structures and properties of homeodomain proteins

Homeobox genes are characterized by a homeodomain of 60 amino. Homeodomain-containing proteins act as transcription factors and bind to specific DNA sequences, thereby regulating the expression of target genes (Bürglin, 1994). It was shown that the homeodomain contains three α-helices, of which helix 1 is separated from helix 2 by a loop whereas helix 2 and helix 3 are separated by a turn.

In *Arabidopsis*, homeobox genes contain not only a sequence encoding the homeodomain, but also a sequence encoding a leucine-zipper domain. The leucine-zipper domain is characterized by a periodic repetition of leucine residues at every seventh position and is linked to the C-terminal of the homeodomain (Ruberti *et al.*, 1991; Mattsson *et al.*, 1992; Schena and Davies, 1992). They were referred to as HD-ZIP, and in *Arabidopsis*, they are designated *Arabidopsis thaliana* homeobox (AtHB) proteins. It was proposed that the leucine zipper motif mediates protein dimerization and only the dimerized protein was shown to be able to bind on DNA (Sessa *et al.*, 1993); dimerization occurs most probably between members of the same HD-ZIP protein family (Sessa *et al.*, 1993; Meijer *et al.*, 1997; Frank *et al.*, 1998; Johannesson *et al.*, 2001). Fourty-two HD-ZIP genes are present in *Arabidopsis* genome (*Arabidopsis* Genome Initiative, 2000) and are grouped in four different families, HD-ZIP I-IV, based on sequence similarities within the homeodomains of deduced amino acid sequences (Sessa *et al.*, 1994; 1998).

1.2.2   Functions of HD-ZIP proteins

HD-ZIP proteins have been shown to be involved in a wide range of processes in plant growth and development. The HD-ZIP II protein AtHB2 is a regulator of cell expansion, as part of the mechanism by which the plant adapts its form in relation to light quality (Carabelli *et al.*, 1996; Steindler *et al.*, 1999). It functions as a negative regulator of gene expression and is involved in mediating specific auxin responses (Steindler *et al.*, 1999). The HD-ZIP III protein AtHB8 has roles in the differentiation of the vascular tissue of the embryo and developing organs (Baima *et al.*, 1995). The HD-ZIP IV protein ANTHOCYANINLESS2 (ANL2) affects anthocyanin accumulation and root development in *Arabidopsis* (Kubo *et al.*, 1999), AtHB10/GLABRA2 control the differentiation of specific cell types: trichomes on leaves and stem, and root hair cells (Rerie *et al.*, 1994; Di Christina *et al.*, 1996).

Functional information on the *Arabidopsis* HD-ZIP I (including AtHB1, AtHB3, AtHB5, AtHB6, AtHB7, AtHB12, AtHB13, AtHB16 *etc.*) proteins suggests that several of these proteins are involved in regulating plant development and plant response to environmental stimuli such as drought, osmolarity stress and ABA (Söderman *et al.*, 1996; 1999; Lee and Chun, 1998). The expression of AtHB7 and AtHB6 in *Arabidopsis* seedlings was markedly induced by water deficit and high salinity treatment as well as by ABA. These inductions

were ABA-dependent, since no increase in *AtHB6* or *AtHB7* transcript was detected in the ABA-deficient mutant *aba-3* when subjected to drought treatment (Söderman *et al.*, 1996; 1999). There are additional reports that the expression of these homeobox genes is dependent on ABA: During transient expression in protoplasts, the AtHB6-binding sequences controlled gene transcription is enhanced in the presence of ABA. Similarly, in transgenic plants, the expression of the *LUC* reporter driven by the *AtHB6* promoter is dependent on ABA (Himmelbach *et al.*, 2002); the transcription of *AtHB7* and *AtHB12* are up-regulated by ABA (Hoth *et al.*, 2002), the promoter of *AtHB12* is inducible in response to ABA treatment (Lee *et al.*, 2001). Furthermore, the induction of *AtHB7* gene expression is reduced in the *abi1* mutant background (Söderman *et al.*, 1996), and the regulation of AtHB7 and AtHB12 by ABA were seriously impaired in the ABA-insensitive mutant *abi1-1* (Hoth *et al.*, 2002). The induction of AtHB6 was impaired in *abi1* and *abi2* mutants (Söderman *et al.*, 1999; Himmelbach *et al.*, 2002). These findings imply that *AtHB6*, *AtHB7* and *AtHB12* are dependent on a functional *ABI1* gene for their transcription and expression. Further studies demonstrated that ectopic expression of AtHB6 decreased ABA-sensitivity in seed germination and in stomatal aperture control (Himmelbach *et al.*, 2002). All these results imply that homeobox genes play important roles in the ABA signal relay leading to the plant's response to drought.

1.2.3   Protein phosphatase ABI1 interacts with *Arabidopsis thaliana* homeobox protein AtHB6

The AtHB6 was identified as a specific interaction partner of ABI1 by using the yeast two-hybrid system (Himmelbach *et al.*, 2002). This interaction was further analysed by *in vivo* studies of transgenic plants as well as by *in vitro* binding assays. It is noteworthy that protein-protein interaction occurs between the PP2C ABI1 and the transcriptional factor AtHB6. Analysis of ABI1 and AtHB6 in the yeast system showed that a critical serine residue within the homeobox of AtHB6 and additional structural features located outside the HD-ZIP motif contributes to the interaction. The interaction of ABI1 with AtHB6 in yeast positively correlated with the PP2C activity of the ABI1 catalytic domain. Inactivation of enzymatic activity by a single point mutation in ABI1 completely abolished the interaction. These analyses indicate that AtHB6 functions as a target for ABI1.

   It is becoming clear that ABA signal transduction is not a simple linear pathway, but has rather complex and redundant branches. The transcriptional factor AtHB6, as one regulator of the ABA signal pathway, can bind to the *cis*-element of its own promoter. It also interacts directly with ABI1. These findings link PP2C with gene regulation. However, what is unexpected is that the over-expression of AtHB6 did not interfere with the expression level of either endogenous *AtHB6* gene or the ABA-regulated genes

Rd29b and *Rab18* (Himmelbach *et al.*, 2002). In view that AtHB6 controls its own expression, it is likely that AtHB6 is not the only factor regulating its promoter activity, and the limiting effects of other factors restricts the alteration of the promoter activity. It is necessary to identify such factors to elucidate the details of regulation pathway of AtHB6.

1.3   Yeast hybrid system in protein interaction assay

Molecular events are usually coordinated by protein-protein interaction. To date the protein-protein interaction analyses have been explored through mechanistic studies that delve into the networks of protein interaction that mediate signal transduction, cell cycle, and gene expression, as well as the biochemical and structural studies of protein interactions, and through cell biological studies that focus on localization and co-localization of proteins in dynamically reorganizing cells. To detect protein-protein interactions, a number of techniques such as the two-hybrid system, far-western analyses, pull down assays, and co-immunoprecipitation are currently used. For certain, these technologies will unravel signaling elements or even signaling complexes.

  The development of yeast two-hybrid system provided a genetic means to identify proteins that physically interact *in vivo*. This system was first established by Stanley Fields and Ok-kyu Song in 1989 (Fields and Song, 1989). Since then, the system has proven to be a highly efficient genetic tool for detecting protein-protein interactions that utilizes the molecular genetics of yeast. It has been used both to screen expression libraries for novel interacting proteins and to study interactions between known partners (Chien *et al.*, 1991). Additionally, this approach has led to the identification of many novel proteins.

  The yeast two-hybrid assay is based on the fact that many eukaryotic *trans*-acting transcription factors are composed of physically separable, functionally independent domains. Such regulators often contain a DNA-binding domain (DBD) and one or more activation domains (AD). The DNA-binding domain binds to a specific enhancer-like sequence, which in yeast is referred to as an upstream activation site (*UAS*; Heslot and Gaillardin, 1992). The activation domains direct the RNA polymerase II complex to transcribe the gene downstream of the *UAS* (Keegan *et al.*, 1986; Hope and Struhl, 1986; Ma and Ptashne, 1987). In the case of most of the native yeast protein (*e.g.* GAL4 protein), the two domains are part of the same protein. If physically separated by recombinant DNA technology and expressed in the same host cell, the DNA-BD and AD peptides do not directly interact with each other and thus cannot activate the responsive genes (Ma and Ptashne, 1988; Brent and Ptashne, 1985). However, if the DNA-BD and AD can be brought into close physical proximity in the promoter region,

the transcriptional activation function will be restored.

In the two-hybrid system, two different cloning vectors are used to generate fusion proteins that potentially interact with each other. One vector is used to generate fusions of special DNA-BD (*e.g.* pGBT9), another vector is used to generate AD-fusion (*e.g.* pGAD424). The recombinant hybrid proteins are co-expressed in yeast and are targeted to the yeast nucleus. An interaction between a bait protein (fused to the DNA-BD) and a prey protein (fused to the AD), either known or from a library of encoded proteins, creates a novel transcriptional activator with binding affinity for the special *UAS* (*e.g.* *GAL4*- or *LexA*-responsive *UAS*). This factor then directs the transcription of reporter genes (*e.g.* *His3*, *LacZ* gene) having this *UAS* in their promoter, making the protein-protein interaction phenotypically detectable (Figure I-4).
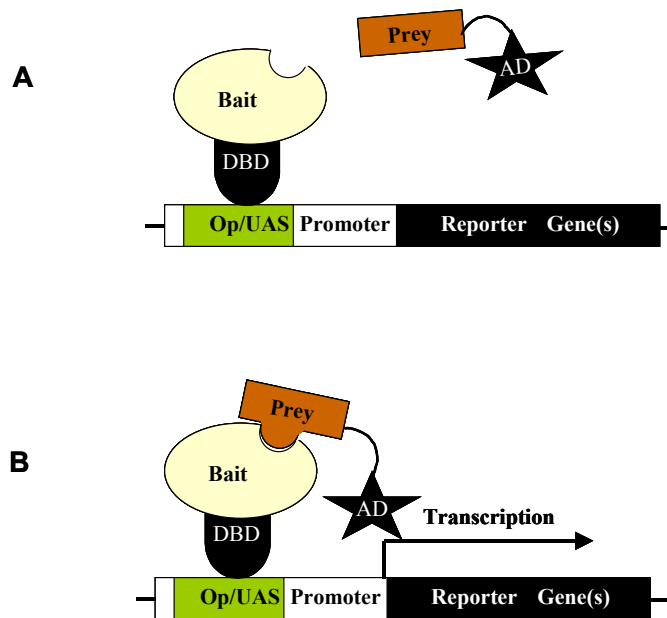
Figure I-4. Schematic diagram of two-hybrid system. A DNA-binding domain (DBD)-fused bait protein of interest and another activation domain (AD)-fused prey protein, either known or selected from a cDNA library, are expressed in yeast. A: If no interaction occurs between the two proteins, the DBD-fusion or the AD-fusion alone cannot activate the transcription of reporter gene. B: If interaction occurs between the two proteins, the DBD and AD are brought together. The interacting pair binds a specific sequence motif (op, operator, or UAS, upstream activating sequence) and activates the transcription of reporter gene.

By using the yeast two-hybrid system, a possible MAPK cascade of *Arabidopsis* was identified (Mizoguchi *et al.*, 2000). Some components in ABA signal transduction pathway were also identified. Four ABI3-interacting proteins were identified by using

ABI3 as the bait protein (Hobo *et al.*, 1999; Schutz *et al.*, 1998; Kurup *et al.*, 2000; Jones *et al.*, 2000). These ABI3-interacting proteins may function with ABI3 within the network of transcriptional regulators in controlling embryo development in *Arabidopsis* (Kurup *et al.*, 2000). The homeodomain protein AtHB6 was postulated to act downstream of ABI1 as a negative regulator of the ABA signal pathway (Himmelbach *et al.*, 2002). The Fibrillin and AtGluRS were identified as the interaction partner of ABI2 (Yang, 2003).

Although the two-hybrid system is useful in detecting interaction between two proteins, this method fails to detect most of contacts between proteins that are part of a larger multiprotein complex such as the RNA polymerase II TFIIH complex (Marinoni *et al.*, 1997) and the yeast cytochrome bc1 components (Hunte *et al.*, 2000). Indeed, each polypeptide, when out of its native complex, could be targeted nonspecifically by other proteins that contact the protein area usually covered by the partner subunits of the complex (Marinoni *et al.*, 1997). On the other hand, one polypeptide belonging to such a larger complex may be insufficient to trap another polypeptide due to the weakness of the interaction; detection of a target protein would thus require multipoint attachment through more than one polypeptide. The three-hybrid system (Tirode *et al.*, 1997) allows the use of two proteins as a bait to identify a third partner by screening available cDNA libraries and the detection of ternary complex formation. It also provides an approach to discover proteins that can inhibit or enhance interaction between two proteins of interest.

The principle of three-hybrid system is similar to two-hybrid system: the physical interaction of DBD-fusion and AD-fusion bring the DBD and AD together to activate the transcription of the reporter, and *vice versa*. What is different is that an additional protein is expressed in the three-hybrid system. This protein might affect the interaction of the other two fusion proteins.

pBridge from CLONTECH company is a powerful vector for the three-hybrid system. pBridge contains two distinct multiple cloning sites (*MCS*) to allow expression of the DBD fusion as well as a third protein (Figure I-5). The *MCSI* is used to clone one gene to generate DBD-fusion protein of interest, and the MCSII can be used to insert another gene to express a third-protein. The expression of the third protein is controlled by a conditional methionine promoter (P_{Met25}) such that it is only expressed in the absence of methionine. This allows expression to be switched on or off by a simple replica plating step. When this vector is used in conjunction with the activation domain (AD) fusion vector, the three-hybrid system is established.

Complex protein interactions can be investigated with the three-hybrid system (Tirode *et al.*, 1997). The third protein in this system can participate in the interaction in several

ways: as a "bridge", by linking two proteins that do not directly interact with each other; as a stabilizer of a weak interaction between two proteins; or as an inhibitor or modifier of one or both of the proteins. Alternatively, a competitor of a two-hybrid interaction can be expressed as the third protein to confirm the specificity of the two-hybrid interaction (Figure I-6).
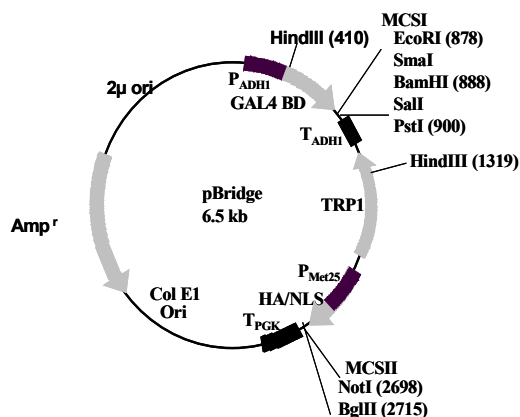
Figure I-5. pBridge three-hybrid vector. A protein of interest is expressed as a fusion to the DNA-BD, while an additional protein of interest is conditionally expressed from the *pMet25* promoter.

1.4 The aim of this work

ABA plays important roles in various aspects of plant growth and development. It seems clear that the ABA response depends on coordinated interactions between positive and negative regulators. Despite significant progress towards understanding ABA signaling, we still have a lot of unclear aspects in the relevant pathways. Identification of novel ABA signal pathway components will be helpful for extending our understanding of the pathways and networks.

The key component of ABA signaling ABI1 regulates several ABA responses. As an interaction partner and target of ABI1, the homeodomain protein AtHB6 regulates part of the ABI1-controlled ABA responses as a transcriptional regulator (Himmelbach *et al.*, 2002). Hence there are more ABI1 targets and interaction partners of AtHB6 to be postulated.

In my dissertation, the major interest was to identify other signaling elements linked to or regulating AtHB6 function. By screening *Arabidopsis thaliana* cDNA libraries using the yeast two-hybrid system for protein interaction with AtHB6, AtHB7 and AtGluRS (*Arabidopsis thaliana* glutamyl-tRNA synthetase) were identified as

interaction partners. The two-hybrid system was further exploited to pinpoint the critical regions that are directly involved in the interaction. Complex protein interactions among AtHB6, AtGluRS and ABI1, ABI2 were investigated using the three-hybrid system. To verify the physical interaction between ABI1 and AtGluRS, the *in vitro* protein affinity interaction assay is performed. In addition, to confirm the *bona fide* protein interaction a co-immunoprecipitation assay is established based on the co-expression of interacting proteins tagged by different epitopes in maize protoplasts.

To clarify the functions of the protein interactions, the ectopic expression of AtGluRS, ABI1 and AtHB6 genes in the protoplast transient expression system and the protein cellular localization by the histochemical GUS staining were studied.
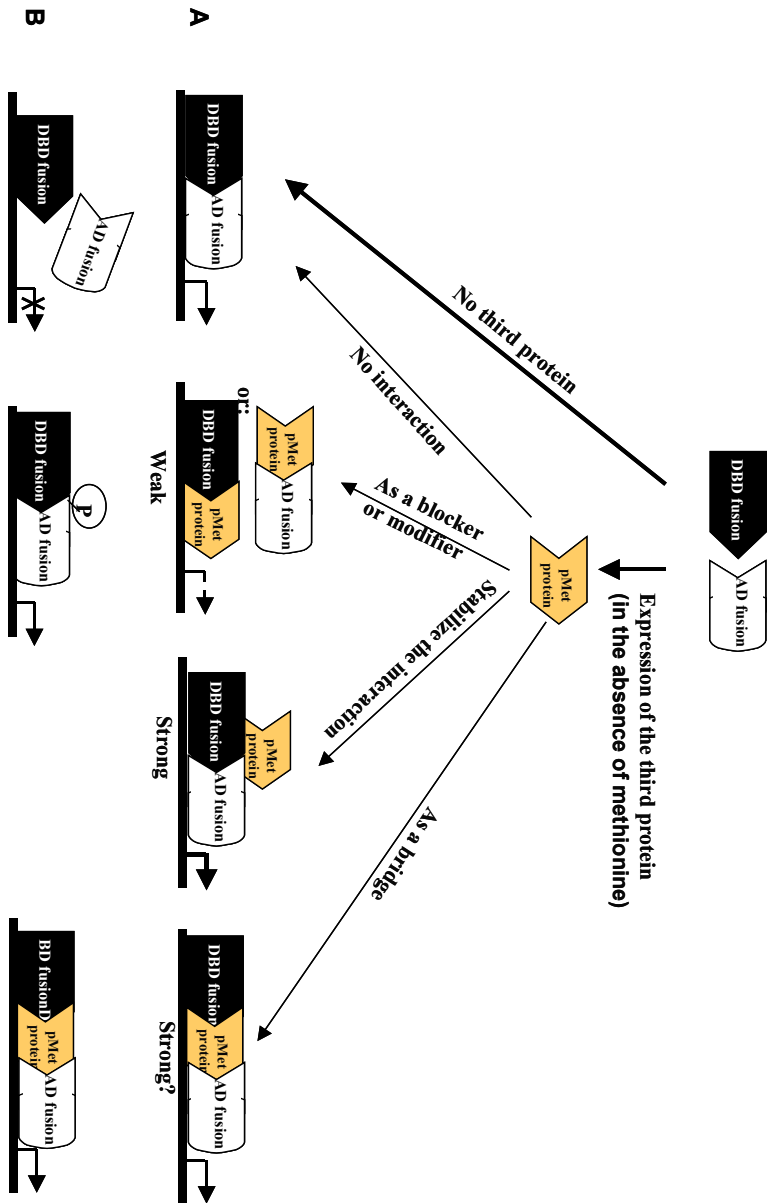
Figure I-6. Complex protein interaction in three-hybrid system. The AD-fusion is expressed from a AD-vector, DBD-fusion and the third protein are expressed from pBridge. A: In the case of that DBD-fusion can interact directly with AD-fusion, the third protein may act as a blocker, inhibiting the interaction; act as a stabilizer, forming complex with the other two proteins; or act as a bridge. B: In the case of no direct interaction between the DBD-fusion and AD-fusion, the third protein can act as a modifier, making the two proteins interact; or act as a bridge, drawing the proteins together.

2   Materials and Methods

2.1   Materials

2.1.1   Microbe materials

Yeast strain

Saccharomyces cerevisiae strain HF7c was provided by CLONTECH (Stehelin & Cie AG, Basel, Switzerland). Genotype of it is: *MATa, ura3-52, his3-200, ade2-101, lys2-801, tr1-901, leu2-3, 112, gal4-542, gal80-538.* The *HIS3* reporter of it is regulated by intact *GAL1* promoter (including the *GAL1 UAS* and *GAL1* minimal promoter), the *lacZ* reporter is controlled by $UAS_{G17-mer(X3)}$ and the extremely weak minimal promoter of the yeast cytochrome *C1* gene. The mutations *trp1* and *leu2* can be used as the selectable markers (Feilotter *et al.*, 1994).

Bacteria strains

The *Escherichia coli* strain DH5α (Stratagene GmbH, Heidelberg) was used for transfomation. Competent cells for plasmid transformation were prepared according to Sambrook and Russell (2001) for routine cloning.

Strain HB101 (Promega) was used for chemical or electroporation transformation. This strain carries the *leuB* mutation and can be used to rescue plasmids bearing the *LEU* marker (like the pGAD plasmid).

Agrobacteria strains

The *Agrobacterium* strain GV3101 pPMP90RK was used for transformation of plant cell cultures with the binary vector pPCV. GV3101 pPMP90 was used for plant cell cultures transformation with binary vector pBI121. Both strains were kindly provided by Dr. Csaba Koncz (Koncz and Schell, 1986).

2.1.2   Plant material

Arabidopsis thaliana

Arabidopsis thaliana Landsberg *erecta* (La-er) were used for producing the protoplasts for protein transient expression. *Arabidopsis thaliana* accessions Reschiev (RLD) were used for crossing to the transgenic plants that are in RLD background. All accessions were received from the *Arabidopsis* Biological Resource Center (ABRC), Ohio, USA.

The transgenic plants with over-expression of GUS and of AtHB6-GUS were generated by Dr. A. Himmelbach. (2002). The AtGluRS sene line (GS) and anti-sense

line (GAS) were generated by Yang (2003).

Arabidopsis (La-er) suspension cultures, were kindly provided by Dr. Csaba Koncz (MPI, Köln).

Maize

Maize *Atfield* was used for producing protoplast for protein transient expression.

2.1.3 cDNA libraries

Ohio library

The cDNA Ohio library of *Arabidopsis* was provided by the DNA Stock Center of the *Arabidopsis* Biological Resource Center, Ohio, USA. The λACT library was made using random-primed mRNA isolated from mature *Arabidopsis* leaves and roots (Elledge *et al.* 1991). The blunted cDNA was ligated to adaptors and inserted into the T-filled XhoI site of λACT. pACT contains the sequences encoding GAL4 AD (aa. 768-881) and the *LEU2* gene for selection in *Leu*⁻ auxotrophic yeast strains.

Clontech library

The *Arabidopsis thaliana* MATCHMAKER cDNA Library was provided by CLONTECH Laboratories, Inc., CA. USA. mRNA was isolated from 3-week-old green vegetative tissue (Bartel *et al.*, 1993). The cloning vector is pGAD10 with GAL4 AD (aa. 768-881) and the *LEU2* gene for selection in *Leu*⁻ auxotrophic yeast strains, and the cDNA was cloned into the EcoRI site in the polylinker of the pGAD10 vector.

2.1.4 Plasmids

Vectors for yeast system

Vectors used for the two- and three-hybrid systems are summarized in Table I-2.

pVA3 carrying the Murine p53 and pTD carrying the SV40 T-antigen are known to interact in the yeast two-hybrid assay and were used as positive control plasmids in yeast two-hybrid system.

Above plasmids were provided by Clontech, except pACT2 which was created in this study by simple digestion of pACT2-GluRS(-20-261) by *Bgl*II and religation. Some basic plasmids maps of these are shown in Figure I-5 and Figure I-7.

Table I-2. Control plasmids for hybrid yeast system

Vector	Description	Selection on SD Medium	References
pGBT9	$GAL4_{(1-147)}$DNA-BD, *TRP1*, amp^r	*-Trp*	Bartel *et al.*, 1993
pGAD424	$GAL4_{(768-881)}$AD, *LEU2*, amp^r	*-Leu*	Bartel *et al.*, 1993
pBridge	$GAL4_{(1-147)}$DNA-BD, *TRP1*, amp^r	*-Trp*	Tirode *et al.*, 1997
pGBKT7	$GAL4_{(1-147)}$DNA-BD, *TRP1*, kan^r, cMyc epitope tag	*-Trp*	CLONTECH
pACT2	$GAL4_{(768-881)}$AD, *LEU2*, amp^r	*-Leu*	Elledge *et al.*, 1991
pVA3	murine p53 in pGBT9	*-Trp*	Iwabuchi *et al.*, 1993
pTD1-1	*SV40 T*-antigen in pACT2	*-Leu*	Li and Frields, 1993
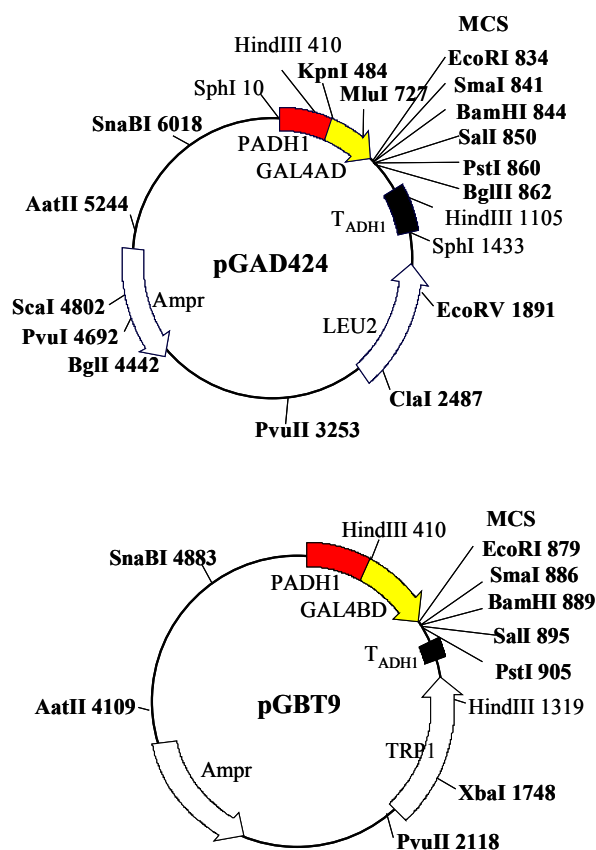
Figure I-7. Schematic diagram of DNA-BD vector pGBT9 and activation vector pGAD424. Unique sites are shown in bold.

Constructs used in *Arabidopsis* cell culture transformation system

The following constructs were used to transform *Arabidopsis* cell cultures:

pMESHI, contains a sequence coding for the cMyc epitope. An intron IV2 from the potato gene ST-LS1 was inserted in this sequence therefore the epitope-tagged protein could be only expressed in plant but not in bacteria (Ferrando *et al.*, 2000; 2001). Suitable restriction endonuclease cleavage sites were placed 5' upstream and 3' downstream of the cMyc codons to allow the cloning of a structural gene modified by cMyc sequences (Ferrando *et al.*, 2001; Figure I-8).

pGIGI, is a derivative of pMESHI produced by *Sal*I digestion and re-ligation (Ferrando *et al.*, 2001).
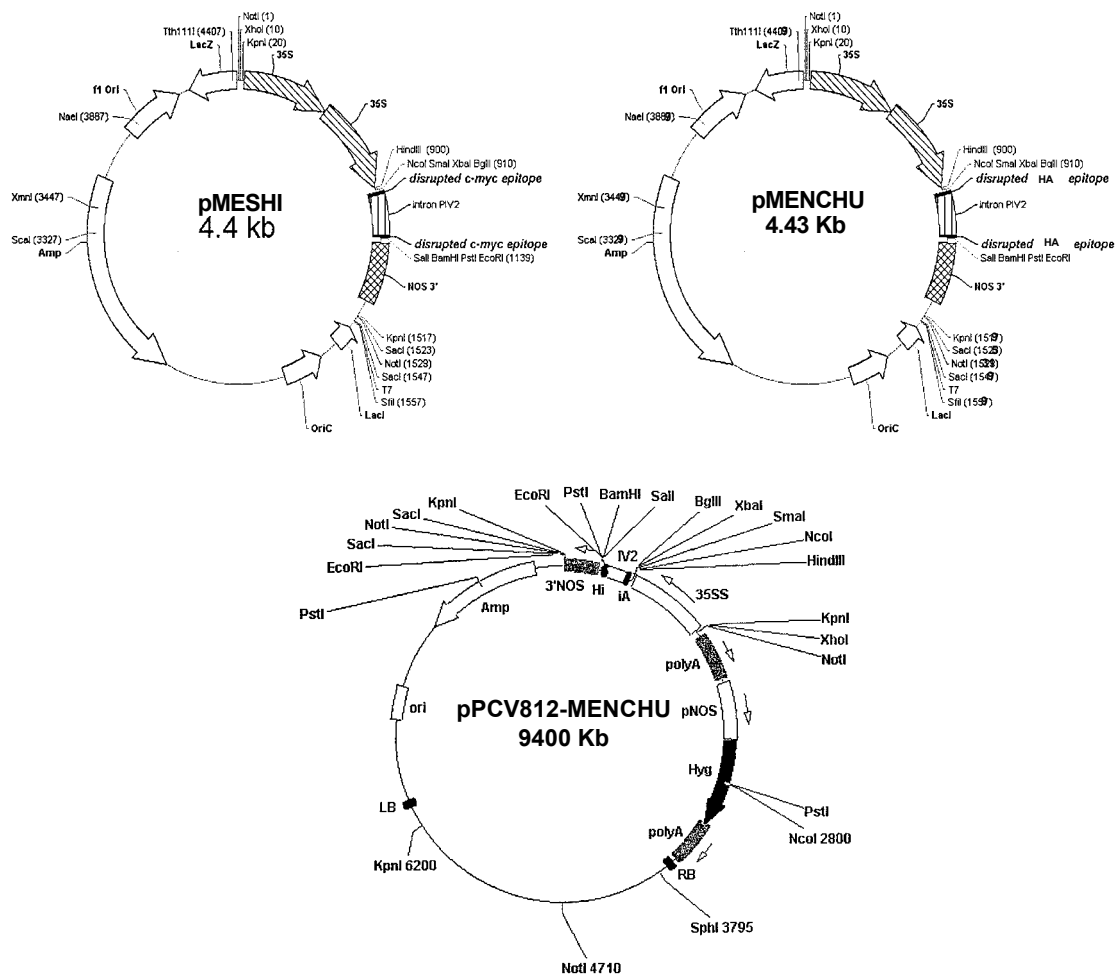
Figure I-8. Schematic diagram of pMESHI, pMENCHU and pPCV-MENCHU. Unique sites are shown in bold.

pMENCHU, contains a sequence coding for the HA epitope. An intron *IV2* from the potato gene *ST-LS1* was inserted in this sequence. Suitable restriction endonuclease cleavage sites were placed 5' upstream and 3' downstream of the HA codons to allow the cloning of a gene with cMyc tag (Ferrando *et al.*, 2000; Figure I-8).

pPCV812-MENCHU, an *Agrobacteria* binary vector (Figure I-8), constructed by Gabino Rios and Alejando Ferrando (2001).

Other constructs

Some constructs created by colleagues and used in my experiment are summarized in Table I-3.

Table I-3. Recombined constructs

Vector	Insert	Constructors or references
pGBT9-ABI1fl	Full length *ABI1*	Leube *et al.*, 1996
pGBT9-abi1fl	Full length *abi1*	Leube *et al.*, 1996
pGBT9-dNABI1	N-terminus deleted (aa 121-434)	Leube *et al.*, 1996
pGBT9-dNabi1	N-terminus deleted (aa 121-434)	Leube *et al.*, 1996
pGBT9-ABI1NAP	Non-active *ABI1* (aa 121-434)	Himmelbach *et al.*, 2002
pGBT9-ABI1 Sac	N-terminus of *ABI1* (aa 1-268)	Leube *et al.*, 1998
pGBT9-ABI2	Full length *ABI2*	Rodriguez, 1998
pGBT9-abi2	Full length *abi2*	Rodriguez, 1998
pGBT9-HB6fl	Full length *AtHB6*	Himmelbach *et al.*, 2002
pGAD424-HB5fl	Full length *AtHB5*	Himmelbach *et al.*, 2002
pGAD424-HB7fl	Full length *AtHB7*	Himmelbach *et al.*, 2002
pQE60-ABI1	Full length *ABI1*	Leube *et al.*, 1996
pQE60-abi1	Full length *abi1*	Leube *et al.*, 1996
pGEX-GluRS	Full length At*GluRS*	Yang, 2003
pBI221-GUS	*CaMV* 35S promoter, full length *GUS*	Jefferson, 1987
pSK-rab18-LUC	Rab18 promoter, full length *LUC*	Hoffmann, 2001
pSK-4xbs-LUC	4 copies of binding sequence (CAATTATTA) in AtHB6 promoter, -46 CaMV 35S core promoter, full length LUC	Hoffmann, 2001

pBI221-AtHB6	CaMV 35S promoter, full length AtHB6	Hoffmann, 2001
pBI221-AtHB7	CaMV 35S promoter, full length AtHB7	Hoffmann, 2001
pBI221-ABI1	CaMV 35S promoter, full length	Hoffmann, 2001
pBI221-GluRS	CaMV 35S promoter, full length AtGluRS	Yang, 2003

2.1.5 Biochemicals and reagents

General chemicals

The chemicals used in this work were of *p.a.* quality unless otherwise stated and were purchased from Fulka/Sigma (Munich), Merck AG (Darmstadt), Roth GmbH & Co. (Karlsruhe), Serva Feinbiochemica (Heidelberg), and Qiagen (Hilden).

Molecular weight standards

DNA markers including 1 kb DNA ladder and 100 bp-DNA ladder were provided by GIBCO/BRL.

λ-*Hind*III was produced by HindIII digestion of λ-DNA.

Prestained protein marker (P7708S) was purchased from New England Biolabs, Frankfurt.

Primers

All primers were synthesized by MWG-Biotech GmbH (Ebersberg, Germany). Details are found in the appendix.

Other biochemicals

Other biochemicals used in the experiments are summarized in Table I-4

Table I-4. Reagents and manufacturer.

Reagents	Manufacturer
Enzymes	
Taq-polymerase, Klenow-fragment polymerase, T4-ligase, restriction enzymes	MBI Fermentas
Pwo-polymerase	Peqlab Biotechnology
Vent-polymerase	New England Biolabs

Calf intenstinal alkaline phosphatase (CIP)	Boehringer Mannheim
M-MuLV reverse transcriptase	Peqlab Biotechnology
Protease inhibitor cocktail	
for plant protein extraction	Sigma
for yeast protein extraction (complete, mini,EDTA free)	Roth GmbH & Co.
Antibodies	
Monoclonal mouse anti-AD, anti-BD, anti-HA, anti-c-myc	Santa Cruz Biotechnology, Inc.
Anti- GST-ABI	Eurogentec
Polyclonal goat anti-mouse IgG peroxidase conjugated antibodies	Santa Cruz Biotechnology Inc.
Goat anti-rabbit IgG alkaline phosphatase conjugated antibodies, goat anti-rabbit IgG peroxidae conjugated antibodies, goat anti-mouse IgG alkaline phosphatase conjugated antibodies	Sigma

2.2 Methods

2.2.1 Cloning techniques

Standard cloning techniques, such as the polymerase chain reaction (PCR), restriction enzyme digestion, DNA fragment end-blunt reaction, de-phosphorylation *via* alkaline phosphatase, DNA ligation, preparation of competent cells, transformation *etc*. were performed according to the protocols of Sambrook and Russell (2001). Some of the methods used in these experiments are described here.

PCR generated fragment for cloning

Pwo or Vent polymerase was used as the DNA polymerase to generate the PCR product and the fragments were further digested by the appropriate enzymes and purified using "QIAquik Gel Extraction" kit (QIAGEN GmbH) prior to cloning.

Generation of blund ends by the large Klenow fragment (polymerase I)

In order to obtain blunt ended DNA fragments, the large Klenow fragment (polymerase I) was used to fill-in the 5'-overhanging end and to remove the 3'-overhanging end taking advantage of its 5'-3' DNA polymerase activity and 3'-5' exonuclease activity. The reaction was performed at 37 $^{\circ}$C for 15 min, and stopped by adding 20 mM EDTA and heating at 65 $^{\circ}$C for 20 min. dNTPs and specific buffer should be included in the reaction according to the requirements of the product.

Dephosphorylation of DNA

The cut vector DNA fragment was normally dephosphorylated before being subjected to ligation in order to reduce vector self-ligation (Manitatis, 1982). 15 µl reaction, including 1-2 µg of linearized plasmid DNA, 1-2 units calf intestinal alkaline phosphatase (CIP) and 1x CIP buffer solution. The reaction was incubated at 37 ^{o}C for 1 hr followed by CIP inactivation with 20 mM EDTA and incubation at 65 ^{o}C 20 min.

2.2.2 Construction of plasmids

C-terminal version (coding for 262-434 aa) of ABI1 named ABI1dN262 was cloned in pGBT9 as follow: The *Xba*I-*Pst*I fragment ABI1dN262 from pGAD424-ABI1fl (Leube *et al.*, 1996) was blunted at the *Xba*I- end and inserted in the *Pst*I and filled-in *Cfr9*I sites of pGBT9.

Constructions of plasmids for yeast hybrid assay and plant *in vivo* expression assay were present in Table I-5, Table I-6, Table I-7 and Table I-8.

Table I-5. Construction of AtHB6 and AtHB7 gene truncations in two-hybrid system

Construction	Preparation of fragments for cloning	
	Vector*	Insert**
pGBT9-HB6dC 269	pGBT9: BamHI, PstI	PCR fragment. Template: pGBT-HB6fl primers: fw: CGGGATCCTAATGATGAAGAGATTAAGTAG (BamHI) rw: AAGGCCTCTGCAGAAATTACCTCCTGGAACC (PstI)
pGBT9-HB6dC 217	pGBT9: BamHI	pGBT-HB6: BamHI, BglII
PGBT9-HB6dC 119	pGBT9: BamHI, PstI	PCR fragment. Template: pGBT-HB6fl Primers: fw: CGGGATCCTAATGATGAAGAGATTAAGTAG (BamHI) rw: AAGGCCTCTGCAGCTGTTTTGTCTTCCACC (PstI)
pGAD424-HB6 dC269	pGAD424: BamHI, PstI	HB6dC269: Product of pGBT-HB6dC269 digested by BamHI and PstI
pGAD424-HB6 dC217	pGAD424: BamHI, PstI	HB6dC217: Product of pGBT-HB6dC217 digested by BamHI and PstI
pGAD424-HB6 dC119	pGAD424: BamHI, PstI	HB6dC119: Product of pGBT-HB6dC119 digested by BamHI and PstI
pGBT9-HB7dC	pGBT9: EcoRI, BglII	PCR fragment. Template: pGAD-HB7fl Primers: fw:ACCGGAATTCATGGAGGTTATGATTCC (EcoRI) rw: AAGGCCTAGATCTCGTCGCCTCTTTTAGCC-3 (BglII)

* The vector plasmid and the restriction enzymes for creating the vector construct are showed. ** The insert was obtained by restriction enzyme digestion or by PCR; the restriction enzymes, the template DNA and the primers are presented. These illustrations are also applicable for the following tables.

Table I-6. Construction of AtGluRS gene truncations in two-hybrid system

Construction	Vector*	Insert**
	Preparation of fragments for cloning	
pGBT9-GluRS dC(-20-261)	pGBT9: BamHI	GluRS(-20~261): Product of pACT-GluRS(-20~261) digested by BglII
pGBT9-GluRS fl	pGBT9: SmaII,BamHI	full-length GluRS: Product of pGEX-GluRS digested by SmaI and BglII
pGAD424-GluRS fl	pGAD424: SmaI, BamHI	full-length GluRS: Product of pGEX-GluRS digested by SmaI and BglII
pACT2-GluRS fl	pACT2: BglII, filled-in	full-length GluRS: Filled-in product of pGEX-GluRS digested by Cfr9I and BglII
pACT2-GluRS(1-110)	pACT2: BglII, filled-in	PCR fragment. Template: pGEX-GluRS Primers: GTSfwd: TGGCCCGGGGGATGGATGGGATGAAGC (XmaI) GTS214r:CGGGATCCTGCAGCTTTCTTTAGCAGGGTTAG (XhoI). filled-in
pACT2-GluRS(1-216)	pACT2: BamHI, XhoI	PCR fragment. Template: pGEX-GluRS Primers: GTSBamHf: CGAATTCCCGGATCCTATGGATGGGATGAAGCTTTC (BamHI), GTS216r: TTGCAGGTCGACGAGTTTAACCTTTCCAATC (SalI)
pACT2-GluRS(1-261)	pACT2 BglII, filled-in	PCR fragment. Template: pGEX-GluRS Primers: GTSfwd: TGGCCCGGGGGATGGATGGGATGAAGC (XmaI), GTS844r: CGGGATCCTGCAGCTTTCTTTAGCAGGGTTAG (PstI). filled-in
pACT2-GluRS(1-445)	pACT2: BamHI, XhoI	PCR fragment. Template: pGEX-GluRS Primers: GTSBamHf: CGAATTCCCGGATCCTATGGATGGGATGAAGCTTTC (BamHI), GTS445r: TTGCAGGTCGACAACTAGGTTTAACCGGCTG (SalI)
pACT2-GluRS(217-445)	pACT2: BamHI, XhoI	PCR fragment. Template: pGEX-GluRS Primers: GTS217f: CGAATTCCCGGATCCCCGGTTTGCTCCAGAGCCAAG (BamHI), GTS445r: TTGCAGGTCGACAACTAGGTTTAACCGGCTG (SalI)
pACT2-GluRS(232-455)	pACT2: BamHI , XhoI	PCR fragment. Template: pGEX-GluRS Primers: GTS232f: CGAATTCCCGGATCCTAAGGCTGCGTTGCTGAACAAG (BamHI), GTS455r: TTGCAGGTCGACAGAAGCTTGCGCTTACTGAG (SalI)
pACT2-GluRS(449-718)	pACT2: BamHI, XhoI	PCR fragment. Template: pGEX-GluRS Primers: GTS449f: TCGGATCCTCTCAGTAAGCGCAAGCTTC (BamHI), GTSSalIr: TTGCAGGTCGACCTCTTCCATCTGG (SalI)

Table I-7. Construction of plasmids for three-hybrid assay

Construction	Preparation of fragments for cloning	
	Vector*	Insert**
pBridge// ABI1fl	pBridge: NotI, BglII	PCR fragment. Template: pGBT-ABI1fl primers: ATAAGAATGCGGCCGCAATGGAGGAAGTATCTCC (NotI), AAGGCCTGGATCCTCAGTTCAAGGGTTTGCTC (BamHI)
pBridge/ HB6dC269// ABI1fl	pBridge// ABI1fl: BamHI, PstI	HB6dC269: Product of pGBT-HB6dC269 digested by BamHI and PstI
pBridge// abi1fl	pBridge: NotI, BglII	PCR fragment. Template: pGBT-abi1fl Primers: ATAAGAATGCGGCCGCAATGGAGGAAGTATCTCC NotI, AAGGCCTGGATCCTCAGTTCAAGGGTTTGCTC BamHI,
pBridge/ HB6dC269// abi1fl	pBridge// abi1fl: BamHI, PstI	HB6dC269: Product of pGBT-HB6dC269 digested by BamHI and PstI
pBridge//H B6dC269	pBridge: NotI,BglII	PCR fragment. Template: pGAD-HB6 Primers: ATAAGAATGCGGCCGCGATGATGAAGAGATTAAGTAG (NotI), AAGGCCTGGATCCGAAATTACCTCCTGGAACC (BamHI)
pBridge/ HB6dC269// HB6dC269	pBridge// HB6dC269: BamHI, PstI	HB6dC269: Product of pGBT-HB6dC269 digested by BamHI and PstI
pBridge//H B7dC	pBridge: NotI,BglII	PCR fragment. Template pGAD-HB7 Primers: ATAAGAATGCGGCCGCCATGGAGGTTCTGATTCC (NotI), AAGGCCTAGATCTCGTCGCCTCTTTTAGCC (BglII)
pBridge/ HB6dC269// HB7dC	pBridge// HB7dC: BamHI, PstI	HB6dC269: Product of pGBT-HB6dC269 digested by BamHI and PstI
pBridge/ HB6dC269	pBridge: BamHI, PstI	HB6dC269: Product of pGBT-HB6dC269 digested by BamHI and PstI
pBridge/ HB6dC269// ABI2	pBridge/ HB6dC269: NotI	PCR fragment. Template pGBT-ABI2 Primers: ATAAGAATGCGGCCGCAATGGACGAAGTTTCTCCTGC (NotI), ATAGATCAGCGGCCGCTCAATTCAAGGATTTGCTC (NotI)
pBridge/ HB6dC269// abi2	pBridge/ HB6dC269: NotI	PCR fragment. Template pGBT-abi2 Primers: ATAAGAATGCGGCCGCAATGGACGAAGTTTCTCCTGC (NotI), ATAGATCAGCGGCCGCTCAATTCAAGGATTTGCTC (NotI)
pBridge/ ABI1dN	pBridge: EcoRI, PstI	ABI1dN: Product of pGBT-ABI1dN digested by EcoRI and PstI
pBridge/ abi1dN	pBridge: EcoRI, PstI	ABI1dN: Product of pGBT-abi1dN digested by EcoRI and PstI
pBridge/ GluRSfl	pBridge: SmaI, PstI	full-length GluRS: Product of pGBT9-GluRS digested by SmaI and PstI
pBridge/ GluRSfl//H B6fl	pBridge/ GluRSfl: NotI, BglII	PCR fragment. Template: pGAD-HB6fl Primers: ATAAGAATGCGGCCGCGATGATGAAGAGATTAAGTAG (NotI), CGGGATCCTGCAGTCAATTCCAATGATCAACG (BglII)

pBridge/ GluRSfl//HB6dC269	pBridge// HB6dC269	full-length GluRS: Product of pGBT9-GluRS digested by SmaI and PstI

Table I-8. Construction of plasmids for in vivo protein expression in plant cell cultures and protoplasts

Construction	Preparation of fragments for cloning	
	Vector*	Insert**
pMESHI-GUS	pMESHI: BamHI, EcoRI	GUS: Product of pB221-GUS digested by BamHI and EcoRI
pMESHI-GluRSfl-GUS	PMESHI-GUS: XbaI, filled-in; BglII	full-length GluRS: Product of pGEX-GluRS digested by SmaI and BglII
pMESHI-GluRS(1-261)-GUS	pMESHI-GUS: NcoI, BglII	PCR fragment. Template: pGEX -GluRS primers: AACCATGGGTCGACAGATGGATGGGATGAAGCTTTC (NcoI), ATAAGATCTGCTTTCTTTAGCAGGGTTAG (BglII)
pMENCHU-GluRSfl	pMENCHU: XbaI, filled-in; BglII	pGEX-GluRS: SmaI, BglII
pMENCHU-GluRS(1-261)	pMENCHU: NcoI, BglII	PCR fragment. Template: pGEX-GluRS Primers: AACCATGGGTCGACAGATGGATGGGATGAAGCTTTC (NcoI) ATAAGATCTGCTTTCTTTAGCAGGGTTAG (BglII)
pMESHI-ABI1	pMESHI: BamHI, filled-in; PstI	ABI1: Product of pGAD424-ABI1 digested by SmaI and PstI
pPCV812-cMyc-GUS	pPCV812-MENCHU: NotI partial digestion	GUS cassette: Product of pMESHI-GUS digested by NotI
pPCV812-GluRSfl-GUS	pPCV812-MENCHU: NotI partial digestion	GluRS cassette: Product of pMESHI-GluRSfl-GUS digested by NotI
pPCV812-GluRS(1-261)-GUS	pPCV812-MENCHU: NotI partial digestion	GluRSdC-GUS cassette: Product of pMESHI-GluRSdC-GUS digested by NotI
pPCV812-GluRSfl	pPCV812-MENCHU: NotI partial digestion	GluRS cassette: Product of pMENCHU-GluRSfl digested by NotI
pPCV812-GluRS(1-261)	pPCV812-MENCHU: NotI partial digestion	GluRSdC cassette: Product of pMENCHU-GluRSdC digested by NotI
pPCV812-ABI1	pPCV812-MENCHU: NotI partial digestion	ABI1 cassette: Product of pMESHI-ABI1 digested by NotI

2.2.3 DNA analysis techniques

Mini-preparation of DNA

The method was according to the protocols described by Sambrook and Russell (2001). 1.5 ml *E. coli* cultures were centrifuged (4000 rpm/ 2 min/ RT) to pellet the cells. The

cells were re-suspended in 100 µl of solution 1 and then mixed gently with 200 µl of solution 2, kept on ice for 5 minutes. 150 µl of solution 3 was added and incubated on ice for 5 minutes. The cell debris was pelleted (14000 rpm/ 5min) and the supernatant was transferred to a new tube. The same volume of phenol/ chloroform/ isoamylalcohol (25:24:1) was added to the supernatant and vortexed for 2 minutes. The mixture was then centrifuged for 5 minutes at 14000 rpm at room temperature. The aqueous phase was transferred to a new tube and the DNA was precipitated on ice with 2 to 2.5 volumes of absolute ethanol for at least 15 minutes. Finally, the plasmid DNA was harvested by centrifugation (14000 rpm/ 10 min), and washed with 80% ethanol, and partially air-dried. The dried DNA was re-suspended in 30 µl 0.1 x TE buffer with 20 ng RNase/ µl (pH 8.0).

Solution 1 (Resuspension buffer):

50 mM	glucose
25 mM	Tris-HCl (Tris (hydroxymethyl) aminomethane, HCl), pH 8.0
10 mM	EDTA (ethylenediamine tetraacetic acid), pH 8.0

Solution 2 (Lysis buffer):

200 mM	NaOH
1%	SDS

Solution 3:

3 M	$KOOCCH_3/HOOCCH_3$, pH 4.8
5 M	Acetic acid

TE buffer:

10 mM	Tris-HCl, pH 8.0
1 mM	EDTA

RNase stock solution:

10 mg / ml	RNase
10 mM	Tris-HCl, pH 7.5

Maxi-prep DNA with silica

This method was a modification of the mini-prep protocol by Myakishev *et al.* (1995).

300 ml overnight cultures were harvested at 5,000 g for 10 min. The pellet was re-suspended in 10 ml of solution 1, and mixed with 20 ml solution 2. After 5 min. 15 ml solution 3 were added and incubated for 5 min. The mixture was centrifuged at 10,000g for 10 min to pellet the cellular debris. The supernatant was cleared by passing through a filter paper. The nucleotides were precipitated by adding 30 ml isopropanol and centrifuged at 4 °C, 10,000 g for 30 min. The pellet was dissolved in 2 ml 0.1xTE. RNA was precipitated by addition of 8 ml 5 M LiCl and centrifugation at 10,000 g for 10 min. The supernatant was supplemented with 10 ml Bind Mix and incubated for 3 min with gentle shaking. Silica was pelleted at 2,000 g for 3 min and washed once with 10 ml 3 M GuSCN (Guanidinthiocyanat)/ 4% (w/v) Triton X-100, and twice with 10 ml

50% ethanol. Finally, the silica pellet was air-dried for about 15 min.

The silica resin having bound DNA was re-suspended in 2 ml 0.1xTE and incubated at 65 ^{o}C for 3 min to elute the DNA. DNA was further concentrated through NaCl/ Ethanol precipitation. The DNA concentration was either measured at 260 nm by photometry (1 OD_{260} = 50 μg/ ml DNA), or estimated by imaging software (Molecular Analyst, BioRad) through comparing with a known DNA marker after agarose gel electrophoresis.

Solutions 1, 2, 3 were as described above.

Binding Mix: for 300 ml overnight cultures.

10 ml	3 M GuSCN
4% (w/ v)	Triton X-100
300 mg	Silica (for high copy plasmid)
or 60 mg	Silica (for low copy plasmid and cosmids)

2.2.4 Protein analysis

Expression and purification of His-tagged proteins in *E. coli*

Expression and native purification of (his)$_6$-ABI1(abi1): This is a modification of the protocol described by the QIA Express Protein Purification System (QIAGEN).

To express (His)$_6$-tagged ABI1 and abi1 in *E. coli*, the *E. coli* strains M15 [pREP4] harbouring the corresponding plasmid (pQE60-ABI1 or abi1) were plated on LB plates. 3 ml LB liquid medium with 100 μg/ml ampicillin and 25 μg/ml kanamycin were inoculated with one colony and incubated overnight at 37 ^{o}C. The starter-cultures were then transferred to 1 litre LB liquid medium with the same antibiotics, incubated at 37 ^{o}C with vigorous shaking until the OD_{600} reached 0.8. To induce the expression of the recombinant protein, an IPTG (isopropyl -D-thiogalactoside) solution was added to a final concentration of 1 mM. After additional 2 hours growing, the cells were harvested by centrifuging at 4000 g for 20 minutes and re-suspended in Sonication buffer (2 ml buffer/ gram cells). After adding 136 μl of 5M NaCl per gram cells, the mixture was incubated on ice for 5 min and sonicated at 30% output, 15 impulses, for 15 sec, 3 cycles using Sonopuls (Bandelin GmbH & Co. KG). Finally, the extract was cleared by centrifugation (30 min, 10,000 g, 4 ^{o}C).

The crude extract was applied to a Ni-NTA column (QIAGEN GmbH, Hilden), which was equilibrated with Buffer A. The column was washed with buffer A till no protein could be detected at 280 nm using HTS 7000 Plus Bioassay Reader (Perkin Elmer) in this buffer. The recombinant protein was then eluted with Buffer B. To remove

imidazole, the recombinant protein was dialyzed against Buffer C for 4 hrs at 4 $^{\circ}$C and then kept at –70 $^{\circ}$C.

Sonication buffer:

50 mM	Na-phosphate buffer (pH 8.0)	1 mg/ml	lysozyme
300 mM	NaCl	10%	glycerol
0.1%	β-mecaptoethanol	10 mM	MgCl$_2$

Buffer A (equilibating and washing):

50 mM	Na-phosphate buffer (pH 8.0)	10 mM	β-mecaptoethanol
300 mM	NaCl	30 mM	Imidazole
0.1%	Tween 20	20%	MgCl$_2$

Buffer B (Elution buffer):

50 mM	Na-phosphate buffer (pH 8.0)	250 mM	Imidazole
300 mM	NaCl	20%	glycerol
0.1%	Tween 20	10 mM	MgCl$_2$
10 mM	β-mecaptoethanol		

Buffer C (Dialysis buffer):

50 mM	Na-phosphate buffer (pH 8.0)	10 mM	β-mecaptoethanol
200 mM	NaCl	30%	Glycerol
0.1%	Tween 20	10 mM	MgCl$_2$

IPTG stock solution:

1 M isopropyl-β-D-thiogalactoside (IPTG) was dissolved in distilled water and filter-sterilized. The stock was stored in –20 $^{\circ}$C.

Expression and purification of GST-AtGluRS fusion proteins in *E. coli*

E.col. DH5α harboring pGEX-GluRS (Yang, 2003) was used for GST-GluRS protein expression.

One single colony was inoculated into 100 ml LB/ampicillin medium and incubated at 37 °C overnight with shaking (200 rpm). The overnight cultures were 10 x diluted and transferred to 1 liter of fresh 2 x YTA medium. Cells were incubated at 26 °C or 30 °C with shaking until the OD$_{600}$ reached 0.8. Fusion protein expression was induced adding IPTG (0, 0.2, 0.5, 1.0 mM) for 2hrs. Cells were harvested by centrifugation and re-suspension in ice cold PBS containing protease inhibitor (50 μl PBS for cells from per ml bacteria culture). After sonication (15 sec, 15 impulses, 30% output and 3 cycles), the suspension was cleared at 4 °C, 12,000 g for 10 min. The supernatant was incubated

with glutathione-sepharose 4B beads (Pharmacia).

Glutathione-sepharose 4B resin was equilibrated twice in PBS. The protein supernatant (~50 ml) was incubated with ~300 μl of equilibrated glutathione-sepharose 4B beads on ice for at least 30 min with gentle agitation. The mixture was drained in a mini-column. The sepharose beads were washed with 3 x 5 ml of PBS. GST-GluRS protein was then eluted by adding 3 x 0.5 ml Elution buffer. Each fraction was analysed by SDS-PAGE. The eluted protein was stored in aliquots at −70 °C.

PBS:

140 mM	NaCl
2.7 mM	KCl
10 mM	Na_2HPO_4
1.8 mM	KH_2PO_4 (pH 7.3)

2 x YTA:

16 g/ l	Tryptone
10 g/ l	Yeast extract
5 g/ l	NaCl
100 μg/ ml	Ampicillin

Elution buffer:

10 mM	Reduced gluthatione
50 mM	Tris-HCl (pH 8.0)

Yeast protein isolation

Extraction of the yeast protein was performed according to the protocol described in the Clontech protocol and mentioned below.

Yeast cells were cultured in 5 ml SD selection medium (Appendix 1) at 30 °C for overnight and then transferred to 50 ml YPD medium (Appendix 1) and incubated for another 4-8 hours at 30 °C until the OD_{600} reached 0.5-1.0. The cells were pelleted (5 min, 1000 g, 4 °C). The pellet was re-suspended in 50 ml cold distilled water and recovered by centrifugation (1,000 g, 5 min, 4 °C). The pellet was immediately frozen in liquid nitrogen.

Preparation of protein extracts by Urea/SDS Method: The cell pellet was thawed in 100 μl of cracking buffer (60 °C) per 7.5 OD_{600} units of cells. The cell suspension was transferred to a new tube containing 80 μl of glass beads (425-600 μm; Sigma # G-8772) per 7.5 OD_{600} units of cells, heated at 70 °C for 10 min and vortexed vigorously for 1 min. After centrifugation at 14,000 rpm for 5 min, the supernatant was transferred to a fresh tube. The extract was analysed by SDS-PAGE or stored in −80 °C.

PMSF (phenylmethl-sulfonyl fluoride) stock solution (100 mM):

0.1742 g PMSF (Sigma # 7626) in 10 ml isopropanol

Cracking buffer stock solution:

8 M	Urea	0.1 mM	EDTA
5 % (w/v)	SDS	0.4 mg / ml	Bromophenol blue
40 mM	Tris-HCl (pH 6.8)		

Cracking buffer:

1 ml	Cracking buffer stock solution		
10 µl	Protease inhibitor (Cocktail, Sigma)		
10 µl	β-Mercaptoethanol	50 µl	100 mM PMSF

Detection of protein-protein interaction *in vitro* (pull-down assay)

5-10 µl Ni-NTA resin was washed with 500 µl binding buffer 3-5 times. The 6 x His-tagged protein was incubated with Ni^{2+}-NTA beads in 100 µl binding buffer (4 °C, 1 hr). Beads were pelleted down at 3000 g shortly and washed in 100 µl binding buffer. The 100 µl supernatant was collected and the binding ability for 6 x His-tagged protein on the resin was monitored using this supernatant later. The protein tested for protein-protein interaction was provided in 100 µl binding buffer and incubated with Ni-NTA resin for 1hr. The beads were pelleted and washed with binding buffer and eluted with the elution buffer. All these steps were processed at 4 °C on a 360 degrees-rolling machine. Fractions from washing and elution steps were stored at –80 °C for further analysis.

Binding buffer:

300 mM	NaCl
10 mM	Mg-acetate
10 mM	Na-phosphate buffer, pH 7.0
5 mM	β-Mercaptoethanol
0.5%	Triton-X
0.05%	SDS

Elution buffer:

300 mM	NaCl
50 mM	Na-phosphate buffer, pH 6.0
250 mM	Imidazol

Protein extraction and co-immunoprecipitation from maize protoplasts

Protein extraction: The maize protoplasts were transfected with plasmid DNA (See section 2.2.9) and the genes were allowed to be transcribed for 12-18 hr. To extract the protein, protoplasts from 20 transfections were collected by centrifugation (80~120 g, 3 min). Protoplasts were lysed in a small volume (*e.g.* 15 µl) of IP-buffer containing 1% SDS on ice for 3 min and then diluted to a final volume of 160 µl with IP buffer to get a final SDS concentration lower than 0,1% (different protein may require different concentration of SDS to get good immunoprecipitation result). The lysate was centrifuged at 14,000 rpm for 2 min, and the supernatant was harvested and used for the immunoprecipitation.

IP-Buffer∗:

150 mM	NaCl	5 mM	EDTA
50 mM	Tris-HCl pH 7.5	1 mM	DTT
1%	Triton X-100	1 mM	PMSF

100 µl /ml   Protease inhibitors "Complete" cocktail (Sigma, P-9599)

∗ prepared freshly; because of the unstabllity of PMSF in aqueous (half-life is about 60 min), additional aliquot of PMSF should be added every 30 min after extraction.

Immunoprecipitation: Immunoprecipitation was performed as described in Figure I-9. Half of each collected fractions were subjected to immunodetection of both the immunoprecipitated protein and the co-immunoprecipitated protein.

Figure I-9. Outline of the procedures for immunoprecipitation

Process	***Fractions collected for protein detection***	***Corresponding to fraction of extract (%)***
Protein crude extracts (150 µl)		
	1/2 of crude extracts (~75 µl) (C)	*50*
1/2 of crude extracts (~75 µl) + 100ng 0.5 µl antibody (*e.g.* anti-HA) shaking slowly at 4 ᵒC for 1-1.5 hr		
+ 10µl Sephorase-protein A Shaking slowly at 4 ᵒC for 1 hr		
	Supernatant (S) (~75 µl)	*50*
Sephorase beads washed with 75 µl IP buffer		
	Supernatant (W) (~75 µl)	*50*
Sephorase beads eluted with 80 µl SDS loading buffer		
	Elution fraction (IP) ~90 µl	*50*

SDS-polyacrylamid gel electrophoresis

SDS-PAGE was performed according to Laemmli (1970).

<u>5 x Loading buffer:</u>

10%	SDS	312.5 mM	Tris-HCl, pH 6.8
25%	β-Mercaptoethanol	0.01%	Bromophenol blue
30%	Glycerol	0.01%	Xylencyanol

<u>Acrylamid solution:</u>

30% (w/v)	Acrylamid
0.8% (w/v)	N,N'-Methylene-Bisacrylamid

<u>10 ml 12% Separating gel solution:</u>

4 ml	Acrylamid solution (Acrylamide/ Bis-acrylamide)
2.5 ml	1.5 M Tris-HCl, pH 8.8
100 µl	10% SDS
3.35 ml	H_2O
50 µl	10% APS (ammonium persulfate)
5 µl	TEMED (N,N,N',N'-Tetramethylethylenamine)

<u>3 ml Stacking gel solution:</u>

0.39 ml	Acrylamid solution	2.3 ml	H_2O
255 µl	1.5 M Tris-HCl, pH 6.8	15 µl	10% APS
30 µl	10% SDS	3 µl	TEMED

<u>SDS gel running buffer:</u>

25 mM	Tris base (pH 8.3)
192 mM	Glycine
0.1%	SDS

Protein detection

Silver staining: Silver staining of SDS-PAGE gels was as described by Blum *et al.* (1987).

The SDS-PAGE was incubated in fixation solution for at least 1 hour to prevent the diffusion of separated proteins. The gel was washed 3 times for 20 min in 50% ethanol and soaked for exactly 1 min in thiosulfate solution then rinsed 3 x 20 sec with water to remove the excess thiosulfate from the gel surface. Following this, the gel was incubated in silver solution for 20 min then rinsed 3 x 20 sec in water. To develop it, the gel was

incubated in developing solution until the bands reached the desired intensity, and then washed with water for 2 x 2 min. The staining reaction was stopped for 10 min in stop solution and equilibration in 50% MeOH. For long time storage, the gel was dried at 80 $^{\circ}$C using a gel dryer (SE 1160, Hoefer Scientific instruments San Francisco).

Fixation solution: 50%   Methanol

                       12%   Acetic acid
                       0.5ml   37% Formaldehyde (/ L)

Thiosulfate solution: 0.2 g/ L $Na_2S_2O_3$

Silver solution: 2 g/ L $AgNO_3$

Developing solution: 6% Na_2CO_3,4 mg/ml $Na_2S_2O_3$

Stop solution: 20%   MeOH, 3% Glycerol

Western-blotting and immunological detection: Proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane (Schleicher & Schuell GmbH) by using an electroblot apparatus (Miniprotean II Biorad) at 190 mA, 59 V for 30 min. The buffer for transferring was:

Transferring buffer: 12 mM   Tris-HCl (pH 8.3)
                       96 mM   Glycine
                       20%   Methanol

   The membrane binding the blotted proteins was stained in Ponceau S (3-hydroxy-4-(2-sulfo-4-(sulfo-phenylazo) phenylazo)-2,7-naphtalene disulfonic acid) shortly to observe the proteins transferred. It was then washed in distilled water and incubated in TTBS buffer containing 5 % nonfat dry milk for 1 hr to block the unspecific binding sites of antibody. The blot was decorated with a primary antibody and incubated for 1 hr with gentle shaking. Depending on the antibody's specificity and affinity, the antibody was diluted to 1:1000 to 1:10000 (*e.g.* 400 ng/ ml) in TTBS (for continuous use stored at 4 $^{\circ}$C and final concentration of 0.01% NaN_3 was added). After the membrane was washed 3 times with TTBS, the secondary antibody conjugated with alkaline phosphatase or peroxidase was added (*e.g.* rabbit IgG- alkaline phosphatase or goat IgG-HRP) and incubated for 1 hr. The secondary antibody was diluted to 1:5000-1:10000 (*e.g.* 20 ng/ ml) in TTBS. For the IgG-AP detection, the membrane was washed 4 x with TTBS and incubated in AP buffer with BCIP/NBT substrate until the bands reached the desired intensity.

   Solution:

Ponceau S solution: 0.3% Ponceau S, 3% TCA

<u>TTBS buffer</u>:

20 mM	Tris-HCl, pH 7.2- 7.4	
150 mM	NaCl	
0.05%	Tween 20	

<u>Alkaline phosphatase buffer (AP)</u> :

100 mM	Tris-HCl, pH 9.5
100 mM	NaCl
5 mM	$MgCl_2$

<u>BCIP stock solution (x100)</u>:   0.5% BCIP (5-bromo-4-chloro-3-indolyl phosphate) in DMF (Dimethylformamid)

<u>NBT stock solution (x100)</u>:   5% NBT (nitro blue tetrazolium) in DMF

Alternatively, for IgG-HRP detection, the membrane was incubated in peroxidase substrate (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce) and monitored with a CCD camera (Hamamatsu digit camera, SimplePCI imaging software).

2.2.5   Cellular localization of GUS-fusion proteins

The binary vector pPCV-MENCHU-GluRS-GUS was transformed into *Arabidopsis* La-er root suspension cultures by *Agrobacterium* strain GV3101 pPMP90RK (Koncz and Schell, 1986) to express the AtGluRS-GUS fusion protein. As negative control the pPCV812-GIGI, and as positive control the pPCV812-MENCHU-GUS for expressing of GUS, were used respectively (Gabino Rios and Alejando Ferrando, 2001).

To survey the influence of AtGluRS on the cellular compartmentation of AtHB6, transgenic plants with over-expression of AtHB6-GUS and with over-expression of AtGluRS were crossed. Roots of 5 days old F1 seedlings and leaf epidermis of 3 weeks old F1 plants were subjected to histological analysis for GUS activity by the modification of the method of Rodrigues-Pousada *et al.* (1993).

For histochemical GUS staining, the *Arabidopsis* root cells or tissues were fixed in fixing solution for 20 min on ice and washed with rinse solution for 3 times. The cells were then incubated in staining solution for 0.5-2 hours until the blue colour appeared. DAPI (4', 6-diamidino-2-phenylindol dihydrochloride) was added to the GUS pre-stained cells or tissues at a final concentration of 3 μM and stained for additional 15 minutes. The cells were then observed for both GUS and nuclear staining by fluorescence microscopy (Zeiss Axioskop).

<u>Fixing solution</u>: Sodium phosphate   150 mM (pH 7.0)

	Formaldehyde 1 %	Triton X-100	0.1 %

<u>Rinse solution</u>:   50 mM Na_2PO_4 (pH 7.2)

	0.5 mM $K_3Fe(CN)6$	0.5 mM	$K_4Fe(CN)_6$

Staining solution:

50 mM	Na_2PO_4 (pH 7.2)	0.5 mM	$K_4Fe(CN)_6$
0.5 mM	$K_3Fe(CN)_6$	2 mM	X-Gluc

DAPI stock:

   300 µM DAPI (4', 6-Diamidino-2-phenylindol dihydrochloride), dissolved in 50 mM sodium-phosphate buffer (pH 7.2) and stored at 4 °C.

2.2.6 Two-hybrid system

The MACHMAKER GAL4 two-hybrid yeast system (Clontech) was used in the experiment.

Carrier DNA

Carrier DNA was prepared according to Schiestl and Gietz (1989). DNA (Sigma D1612, deoxyribonucleic acid sodium salt from salmon testes) was dissolved in TE (pH 7.5) at a concentration of 10 mg/ml by shaking for 1 hr at room temperature. Following that, 2 x 30 sec sonication processed. The DNA solution was extracted once with phenol (pH 8.0 TE saturated), once with phenol:chloroform (1:1) and once with chloroform. The DNA was then precipitated by adding 1/10 vol of 3 M sodium acetate (pH 6.0) and 2.5 volumes of ice-cold ethanol (100%). The precipitate was collected by centrifugation and

Figure I-10. Carrier DNA prepared for salmon testes DNA. The molecular weight is ranged in size from 1-10 kb.

washed with 75% ethanol, partially dried and then dissolved in TE. This solution was denatured in boiling water bath for 20 min and then immediately cooled in an ice-water bath. Carrier DNA was stored in aliquots at –20 °C. Prior to transformation, the carrier DNA was heat denatured and chilled in ice water. DNA prepared in this way had an average size of around 6 kb as judged from an ethidium bromide stained agrose gel after seperation and ranged in size from 1-10 kb (Figure I-10). The efficiency of yeast transformation mediated by LiAc-PEG using this carrier DNA was around $6.6x10^4$ to $1.0x10^5$ transformants/ µg plasmid.

Yeast competent cells

One yeast colony was inoculated in 1 ml of YPD or SD liquid medium (appendix 1). Cells were transferred to 30 ml YPD (or 150 ml for large scale transformation) or the

appropriate SD medium and incubated overnight at 30 ^{o}C with shaking. Overnight cultures were cultivated in 250 ml YPD (or 1000 ml for large scale transformation) to arrive OD_{600} 0.4-0.6 (in log phase). Cultures were centrifuged (2000 g, 5 min) and washed with ice-cold sterile distilled water. The cell pellet was dispersed in 1.5 ml (or 10 ml for large scale transformation) freshly prepared 1xTE/1xLiAc.

Solution:

10xTE: 0.1 M Tris-HCl, 10 mM EDTA, pH 7.5

10xLiAc: 1 M lithium acetate acid, pH 7.5 (adjusted by acetate acid)

1xTE/1xLiAc: (10 ml)

10 x TE buffer	1 ml
10 x LiAc	1 ml
ddH$_2$O	8 ml.

   This solution should be prepared freshly just prior to be used.

Small scale yeast transformation

100 ng plasmid and 100 µg carrier DNA were mixed in a sterile 1.5 ml tube, then 100 µl freshly-made yeast competent cells (HF7c) and 0.6 ml sterile PEG/LiAc solution were added and mixed by vortexing at high speed for 10 sec. The mixture was incubated at 30 ^{o}C for 30 min with shaking at 200 rpm and 70 µl DMSO were added. After 15 min heat-shock at 42 ^{o}C in water bath, the mixture was chilled on ice for 2 min. Cells were pelleted (4000 rpm, 2 min) and washed with 1 ml 1xTE. Finally the cells were re-suspended in 0.5 ml TE. 100 µl suspension was plated on selective SD agar plates and incubated at 30 ^{o}C until colonies appeared (2-4 days).

   The colonies growing on the plate were counted to give the colony forming units (cfu) and transformation efficiency per µg plasmid was calculated.

PEG/LiAc solution (10 ml):

10 x TE buffer	1 ml
10 x LiAc	1 ml
50% PEG4000	8 ml (final conc.: 40%)

Large scale yeast transformation for screening

10 ml yeast competent cells (HF7c), prepared as described above, were mixed with 100 µg library plasmid DNA, 20 mg of carrier DNA and 60 ml of sterile PEG/LiAc solution by vortexing at high speed for 30 seconds. The mixture was incubated at 30 ^{o}C for 30 minutes with shaking at 200 rpm. 7 ml of DMSO was added and mixed by hand. After

15 minutes heat-shock at 42 ^{o}C in a water bath, the cells were chilled on ice for 2-5 minutes. The cells were then harvested (1,000 g, 5 minutes) and washed in 20 ml 1xTE. Finally, the cells were re-suspended in 10 ml of sterile 1xTE buffer. Transformed cells were plated on plates SD-Trp-Leu-His (SD lacking tryptophan, leucine and histidine) with 8 mM 3-AT (3-amino-1,2,4-triazole; Sigma) to reduce the growth of background colonies, and incubated at 30 ^{o}C until the colonies appeared (normally 4-10 days). The growing colonies were chosen as the putative *His3*-positive candidates.

Analysis and verification of putative positive candidates

Preliminary elimination of false positives: The putative positive colonies were streaked on SD-Leu-Trp-His plates to verify the *His3*-reporter gene expression, and on SD-Leu-Trp to test the *LacZ*-reporter gene expression by colony-lift filter assay. 3 to 6 single positive colonies of each candidate were re-streaked and retested for their His3 and LacZ phenotypes.

Plasmid isolation from yeast: One or several *His3*$^+$ and *LacZ*$^+$ positive fresh colonies (2-4 mm) were inoculated in 3 ml SD selection medium and incubated at 30 ^{o}C overnight with shaking at 200 rpm. 1.5 ml overnight cultures were spun down in a 1.5 ml eppendorf tube (14,00 rpm, 5 sec). The pellet was re-suspended in 200 µl breaking buffer. 200 µl of acid-washed glass beads (0.45-0.55 mm, Sigma) and 200 µl phenol/chloroform/isoamylalcohol (25:24:1) were added to the suspension and the mixture was then vortexed at high speed for 2 min. The extract was centrifuged (14,000 rpm, 5 min) and the upper aqueous phase was transferred to a fresh eppendorf tube. 1/10 volume of 3 M Na-Acetate (pH 6.0) and 2.5 volumes of ethanol were added to precipitate the DNA. After 15 min incubation on ice, the DNA was recovered by centrifugation (14,000 rpm, 15 minutes). The pellet was washed with 80% ethanol, dried, and finally dissolved in 20-30 µl of sterile water.

<u>Breaking buffer:</u>

2%	Triton X-100	10 mM	TrisCl, pH 8.0
1%	SDS	1 mM	EDTA
0.1M	NaCl		

Analysis of the AD/library inserts: The AD/library insert in the plasmid from *His3*-and *LacZ*-positive yeast transformants was PCR-amplified by using AD forward primer (TACCACTACAATGGATG) and AD reverse primer (GTTGAAGTGAACTTGCG). The PCR products were digested with restriction enzymes for characterization. This step was used to eliminate duplicate colonies bearing the same AD/library plasmid.

Rescueing of the AD-plasmid: HB101 electroporation competent cells (prepared by the method of Dower *et al.*, 1988) were thawed on ice. 10 µl of yeast plasmid DNA and 40 µl of competent cells were mixed and transferred to a pre-chilled cuvette (0.2 cm electrode gap). Electroporation was performed by Electroporator Easyject (EquiBio) at 2500 V, 25 µF, 201 Ω, and time constant = 5 sec. Following the pulse, the cells were immediately removed from the electrodes and mixed with 1ml of outgrowth medium SOC (see Appendix). The samples were incubated with shaking at 37 ^{o}C for 1 hr. Cells were pelleted (4000 rpm, 2 minutes) and washed with 1xTE (pH 8.0). The pellet was re-suspended in 100 µl TE and plated on M9 (see Appendix 1) /ampicillin agar medium supplemented with appropriate amino acids (*e.g.* omitted Leu for selecting AD-plasmid). These plates were incubated in 37 ^{o}C for 2 days to obtain the transformants.

Sequence AD/library inserts and database analysis: Inserts of the positive AD/library plasmids were sequenced by MWG company. Sequences were compared with DNA databases, *e.g.* GenBank, by using the BLAST programme (Altschul *et al.*, 1997). The open reading frame (ORF) of the insert fused to the GAL4 AD sequence was determined.

β-Galactosidase assay

Colony-lift filter assay: This method was according to the CLONTECH protocol.

A clean, dry filter was placed over the surface of fresh colonies grown for two days on SD medium. The filter was gently rubbed with the side of the forceps to help colonies adhere to it. The filter was transferred to a pool of liquid nitrogen frozen for 10 seconds and then was allowed to thaw at room temperature. This filter was placed on the pre-soaked waterman #5 filter saturated with Z-buffer/X-Gal solution and incubated at 30 ^{o}C.

Z-buffer (pH 7.0):   (1L)

16.1 g	$Na_2HPO_4{}^.7H_2O$	0.75 g	KCl
5.5 g	$NaH_2PO_4{}^.H_2O$	0.246 g	$MgSO_4{}^.7H_2O$

X-gal stock solution:

X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was dissolved in DMF (N,N-dimethylformamide) at a concentration of 20 mg/ml and stored at –20 ^{o}C.

Z-buffer/X-gal solution:

100 ml	Z buffer
0.27 ml	β-mercaptoethanol
1.67 ml	X-gal stock solution

Quantitative β*-galactosidase assay:* 1 ml of overnight cultures grown in proper selective SD medium were transferred to 4 ml YPD and incubated at 30 $^\circ$C with shaking until the cells reached in mid-log phase (OD$_{600}$ of 1 ml = 0.5-0.8). 1 ml of these fresh cultures was centrifuged (14,000 rpm, 30 seconds) and washed in Z-buffer. Pellets were re-suspended in 100 µl of Z-buffer, frozen in liquid nitrogen, and thawed in a 37 $^\circ$C water bath. The freeze/thaw cycle was repeated two times. 700 µl of Z-buffer with β-mercaptoethanol and 160 µl of ONPG in Z-buffer were then added to the reaction. The tubes were incubated in 30 $^\circ$C. After the yellow colour developed, 0.4 ml of 1 M Na$_2$CO$_3$ was added to stop the reaction and the elapsed time in minutes was recorded. The reaction solution was cleared by centrifugation (14,000 rpm, 10 minutes) and the supernatant was transferred to cuvettes. The OD$_{420}$ of the samples was determined relative to the blank.

 1 unit of β-galactosidase is defined as the amount of enzyme that hydrolyzes 1 µmol of ONPG to o-nitrophenol and D-galactose per minute per cell (Miller, 1972; 1992):

Solutions:

ONPG:
4 mg/ ml ONPG (O-nitrophenyl β-D-galactopyranoside) were dissolved in Z-buffer pH 7.0.

Z buffer with β-Me: 100 ml   Z buffer, pH 7.0
                          0.27 ml   β-mercaptoethanol

2.2.7 Three-hybrid yeast system

The yeast three-hybrid system was used to study interactions among three proteins. The AD/prey vector and pBridge vector were introduced into the yeast strain HF7c. pBridge was used to express the DBD-fusion protein and another "third protein" controlled by conditional active methionine promoter *pMet$_{25}$*. The procedures for transformation, *His3* report gene test, and *LacZ* report gene test, were performed as those described for the two-hybrid system. 0, 0.15 and 1.0 mM of methionine contained in SD medium were used to control the expression of the third protein.

2.2.8 *Agrobacterium*-mediated plant cell transformation

Transformation of *Agrobacterium*

Preparation of competent Agrobacterium cells: Single *Agrobacterium* colony (GV3101 with pMP90RK) was incubated in LB medium with appropriate antibiotics (rif[50], kan[50]) at 30 $^\circ$C with vigorous shaking for overnight. 0.5 ml of the pre-culture was transferred to

100-150 ml LB (with rif^{50}, kan^{50}) in 1 L flasks and incubated at 28-30 ^{o}C with vigorous shaking until OD$_{600}$ arrived 0.5-1.0. The cells were harvested by centrifugation (5000 rpm, 20 min, 4 ^{o}C) and sequentially washed in 150 ml, 100 ml, 20 ml of ice-cold 10% glycerol. Finally, the cells were re-suspended in 7.5 ml 10% glycerol and aliquoted to 1.5 ml eppendorf tubes in liquid N$_2$ and stored at -80 ^{o}C.

Electroporation of Agrobacterium: 100 ng of plasmid mixed with 100 µl *Agrobacteria* competent cells were transferred to a pre-cooled cuvette with 0.2 cm gap and electroporated by Electroporator Easyject (EquiBio) at V= 2.5 kV, C= 25 µF, R= 700 Ω with the expected time constant of 18 ms. Cells were re-suspended in 800 µl YM medium (Appendix 1) and incubated at 30 ^{o}C for 3 hr with shaking. Transformants were plated on YM-plates with the appropriate selection antibiotics and incubated at 30 ^{o}C for 2-3 days.

Preparation of *Arabidopsis* suspension cell cultures

Arabidopsis root cell suspensions were sub-cultivated weekly in modified MS medium (Appendix 1) by mixing 15 ml old cell cultures with 35ml fresh medium in 250 ml flasks. Cells were cultivated at 22 ^{o}C, 150 rpm, in dark (Koncz *et al.* 1984).

Transfection of *Arabidopsis* suspension cell cultures

Agrobacterium strains carrying binary vectors were cultivated in selective YEB medium (Koncz *et al.* 1994; Appendix 1) until OD$_{600}$ reached 0.8~1.0 (1x10^9 cells/ ml), collected by centrifugation, and re-suspended in 0.1 *vol* of cell culture medium (1x10^{10} cells/ ml). 1 ml of this *Agrobacterium* cell suspension was routinely used for infection of 50ml freshly sub-cultivated *Arabidopsis* cell suspension (Koncz *et al.* 1994). After 3 days co-cultivation, claforan (500 µg/ ml, Hoechst, Germany) was added to arrest bacterial growth, and 2 days later the *Arabidopsis* cells were collected for GUS staining or preparation of protein extracts. To maintain stable transformed cell lines expressing the T-DNA-encoding a hygromycin resistance marker and epitope labelled genes, 15 ml of *Arabidopsis* cells were sub-cultivated weekly in 35 ml fresh cell culture medium containing 15 µg/ ml hygromycin as well as 500 µg/ ml claforan (Mathur and Koncz, 1998).

2.2.9   Transient expression in protoplasts

Protein expression in *Arabidopsis thaliana* protoplasts

The isolation of protoplasts was performed according to a modified protocol from Abel and Theologis (1994) and the expression analysis was carried out by a modification of

the method of Hoffmann (2001).

Preparation of protoplasts from Arabidopsis leaves: 1-2 g of leaf material were collected from 4-week old *Arabidopsis* La-er seedlings and washed in deionised water. The drained leaves were then incubated in 10 ml enzyme solution for about 3-4 hours at 28 $^{\circ}$C with gently shaking (30 rpm). The protoplasts were harvested by passing through a 150 µm-mesh nylon net. After centrifugation at 60 g for 2 min, the supernatant was discarded and the protoplast pellet was carefully re-suspended in Washing solution 1 by gently rolling the tube by hand. Subsequently, the pellets were washed in washing buffer 2 and Ma-Mg solution. After the protoplasts were re-suspended again in Ma-Mg solution, the concentration of protoplast was determined. To visualize the living protoplasts (Nunberg and Thomas, 1993), 2 µl of FDA (fluorescein-diacetat) solution was added to 50 µl of the suspension, and the living protoplasts were counted by monitoring the emitted fluorescent light in fluorescent microscope (Zeiss Axioskop). The optimal concentration of protoplasts for transient expression was found at 0.5×10^6/ ml.

Enzyme solution:

1%	Cellulase R-10
0.25%	Macerozyme R-10
400 mM	Mannitol
8 mM	CaCl2 1 % BSA
5 mM	MES (pH 5.6, adjusted by KOH)

FDA solution: FDA 1 mg/ ml in Acetone

Washing solution 1:

167 mM	Mannitol
133 mM	CaCl$_2$

Washing solution 2:

333 mM	Mannitol
67 mM	CaCl$_2$

Ma-Mg solution:

400 mM	Mannitol
15 mM	MgCl$_2$
5 mM	MES (pH 5.6, adjusted by KOH)

Protoplast transformation by PEG: About 20~30 µg of each plasmid for reporter constructs and for test construct were mixed with 100 µl protoplast suspension. After adding an equal volume (volume of plasmids + protoplasts) of PEG solution, the tubes were mixed immediately by inverting 3-4 times and incubated at room temperature for 3-5 min. 1 ml of sterile filtrated WI solution was then added to the tube and centrifuged (2000 rpm, 2 min). The protoplasts washed with 1ml WI and re-suspended in

appropriate volume of WI. The samples were incubated in dark at 25 °C with shaking (30 rpm) for 4-6 hr. ABA induced gene expression was assayed by incubation the samples with 30 µM ABA for additional 16hr.

PEG solution:

300 mM   CaCl$_2$
40%   PEG-4000
0.5%   MES-KOH (pH 5.8)

WI:

500 mM   Mannitol
20 mM   KCl
1%   BSA
5 mM   MES-KOH (pH 5.8)

Protein expression in maize protoplast

Preparation of protoplasts from etiolated maize leaves

Hybrid maize *Atfield* seeds (Saaten Union) were soaked in water for 10 hr, sowed in vermiculite and grown at 25 °C in dark for 10-12 days until the second leaves reached 10-15 cm. The second leaves were then harvested for the protoplasts preparation. The middle parts (6-8 cm) of these leaves were cut into 0.5 mm strips with sharp blade to avoid bruising. Leaf strips embedded in 10 ml enzyme solution were vacuumed for 20 min for infiltration. The digestion was continued for another 3 hr with gentle shaking (40 rpm) on a platform shaker. The protoplasts were harvested by passing through a 150 µm-mesh nylon net and centrifuged at 130 g for 2 min. The protoplast pellet was washed twice in electroporation buffer. Protoplasts were re-suspended again in electroporation buffer. The density of the protoplasts was determined by monitoring the emitted fluorescent light in fluorescent microscope after adding 2 µl FDA solution to 50 µl of the protoplast suspension. The density of protoplast for transient expression was adjusted to an optimal value of $1\text{-}2\text{x}10^6$/ ml.

Transformation through electroporation: Transfection of $1.5\text{x}10^5$ protoplasts in 150 µl with 25-60 µg of plasmid DNA per electroporation was done in pre-cooled cuvette with 0.2 cm gap by using an electroporator BTX T-820. The electroporation condition was 5 msec, 155~160 V, one pulse. After electroporation, the samples were kept on ice for 10 min and then re-suspended in 400 µl of Incubation solution, incubated at 28 °C in dark with shaking (40 rpm) for 15-20 hr. ABA induced gene expression assay was performed by adding 30 µM ABA to the samples after an initial 4-6 hr incubation.

Solutions:

Enzyme solution:

1.5%   Cellulase RS
0.3%   Macerozyme R10

0,6 M	Mannitol
10 mM	MES (pH 5.7); heated at 50-55 ^{o}C for 10 min to inactivate protease
1 mM	CaCl$_2$
5 mM	β-mercaptoethanol
0.1%	BSA

Electroporation solution:

0.6 M	Mannitol
4 mM	MES (pH 5.7)
20 mM	KCl

Incubation solution:

0.6 M	Mannitol
4 mM	MES (pH 5.7)
4 mM	KCl

Assay for reporter gene expression

The transfected protoplasts were pelleted at 60 g for 2 min and dissolved in 105 µl of CCLR buffer, vortexed, and kept in -80°C for 1 hour or in liquid nitrogen for several min. Cell debris was removed by centrifugation and the cleared extract was used for enzyme measurements.

CCLR:

25 mM	Tris-phosphate buffer (pH 7.8)
2 mM	EDTA
2 mM	1,2-diaminocyclonhexan-N,N,N',N'-tetraessig acid
10% (v/v)	Glycerol
1%	Triton-X-100

β-D-glucuronidase activity assay: 50 µl of extract was mixed with 100 µl GUS assay buffer. The fluorescent glucuronidase measurement was carried on black micro-plate (Nunc GmbH & Co. KG) in the HTS 7000 plus Bioassay Reader from Perkin Elmer using excitation filter 360 nm and emission filter 465 nm.

GUS assay buffer:

0.2 mM	MUG (4-Methylumbelliferyl-β-D-glucuronid)
50 mM	Na-phosphate buffer (pH 7.0)
10 mM	Na$_2$EDTA
0.1%	Triton X-100
(1 mM	DTT)

Firefly luciferase assay: The luciferase activity in protein extracts was measured according to Luehrsen *et al.* (1992). 50 µl of extract for each sample were assayed for the luciferase activity. Luciferase activity was measured using a flash'n glow Luminometer (Berthold) in light-units (lu) per 90 seconds. Before measurement, the substrate buffer was prepared. The Luminometer automatically injected 100 µl of substrate buffer into each sample tube for the reaction.

Substrate buffer:

20 mM	Tricine (pH 7.8, adjusted by NaOH)
2.7 mM	$MgSO_4$
0.5 mM	EDTA
33.3 mM	DTT
0.53 mM	ATP
1.07 mM	$(MgCO_3)_4Mg(OH)_2\ 5H_2O$
0.27 mM	Coenzyme A
0.47 mM	D-luciferin (PJK GmbH)

3   Results

3.1   Identification of interaction partners of AtHB6

It has been reported that the homeodomain protein AtHB6 is a target of the PP2C ABI1 and is involved in ABA signal transduction (Himmelbach *et al.*, 2002). The data indicated that there are additional components required for proper ABA signal transduction. Hence, it was of interest to determine if other proteins interact with AtHB6. The two-hybrid yeast system (Bartel and Fields, 1995) was therefore exploited to screen for additional interaction partners of AtHB6.

3.1.1   Different versions of bait proteins

Preliminary experiments revealed that AtHB6 fused to the GAL4 DNA-binding domain (DBD) as the bait provided very strong transcriptional activation of *lacZ* and *His3* reporters in the absence of any activation domain (AD) fusion (Figure I-11). In order to reduce unwanted transcriptional background activation, three short cDNA fragments of AtHB6 were inserted into pGBT9 (Figure I-12). The DBD-fusions created are pGBT9-HB6dC269, pGBT9-HB6dC217 and pGBT9-HB6dC119, coding for the fusion
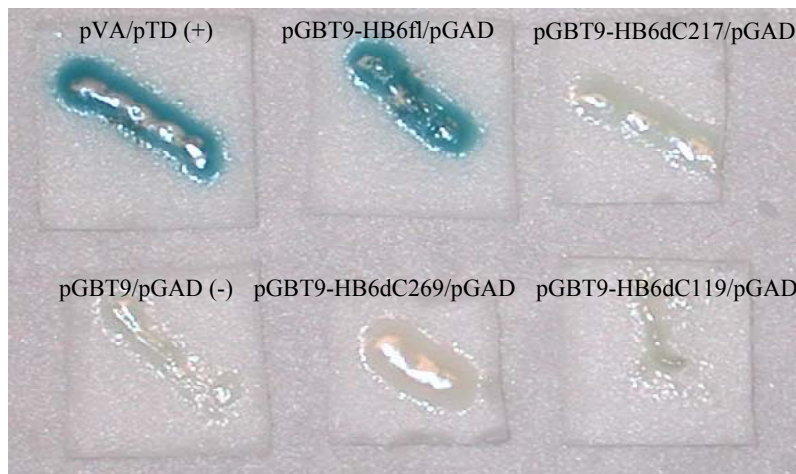
Figure I-11. Autonomous transcriptional activation of different DBD-AtHB6 versions tested by colony-lift filter assay. Different versions of AtHB6 in pGBT9 were co-transformed with pGAD424 to yeast HF7c, and fresh colonies were used for the filter lift assay. Co-tansformant pVA/ pTD and pGBT9/ pGAD424 were used as the positive and negative control, respectively, for *LacZ* expression.

proteins DBD-AtHB6dC269 (amino acids 1-269 of AtHB6), DBD-AtHB6dC217 (amino acids 1-217 of AtHB6) and DBD-AtHB6dC119 (amino acids 1-119 of AtHB6), respectively.

These DBD-fusions were further tested for the activation of reporter gene expression to ensure that they were suitable to be used as baits for library screening. The yeast HF7c cells co-transformed with pGAD424 and pGBT9-HB6fl, pGAD424 and pGBT9-HB6dC217, pGAD424 and pGBT9-HB6dC269 or pGAD424 and pGBT9-HB6dC119 were subjected to the colony-lift filter assay for β-galactosidase. The HF7c cells carrying pGBT9/pGAD424 or pVA/pTD were used as negative and positive controls, respectively. The expression of the *lacZ* reporter gene was indicated by the formation of blue color due to the β-galactosidase activity of yeast cells. Figure I-11 shows that yeast HF7c harboring pGAD424 / pGBT9-HB6fl exhibited blue color as strong as that of the positive control pVA/pTD, while no blue color was presented in the yeast HF7c carrying pGAD424 / pGBT9-HB6dC217, pGBT9-HB6dC269, or pGBT9-HB6dC119. This indicates that the full-length AtHB6 fused to DBD autonomously activated the *lacZ* gene, and the ability of the truncated versions to activate reporter transcription was reduced to a very low background level. Thus, these truncated versions could be used as baits to screen the library.
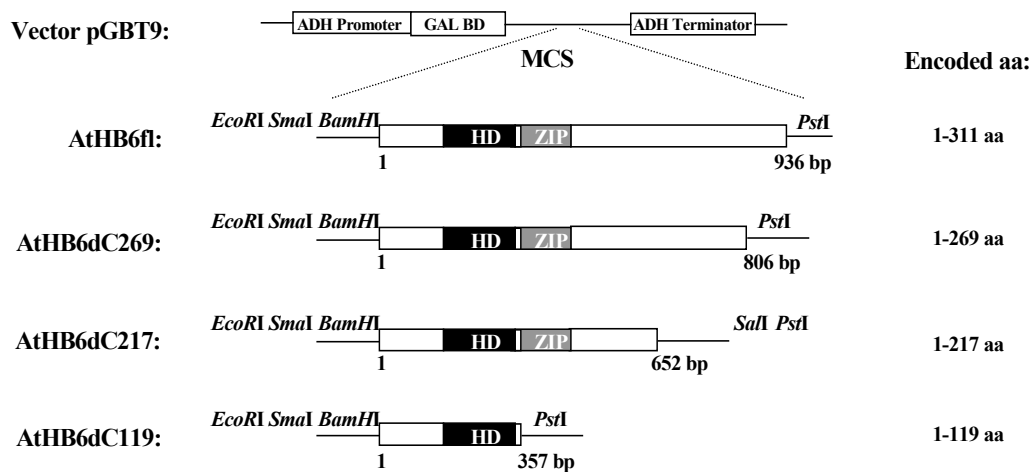
Figure I-12. Schematic diagram of constructs of different AtHB6 versions in pGBT9. Regions coding for homeodomain (HD) and leucine zipper (ZIP) in AtHB6, and the multiple cloning sites in the pGBT9 vector are presented. The nucleotide length as well as the numbers of amino acid (aa) encoded by the cDNA constructs of AtHB6 is given.

3.1.2 Library screening with the yeast two-hybrid system

Positive candidates

To identify the proteins interacting with AtHB6, the three deleted versions generated were used as bait. Prior to screening, the three DBD-constructs, pGBT9-HB6dC269, pGBT9-HB6dC217 and pGBT9-HB6dC119 were introduced into yeast HF7c cells auxotrophic for tryptophan and leucine, and then plated on selective media without tryptophan. The well-growing transformants were then selected and used for library screening.

The competent yeast cells harboring pGBT9-HB6dC269 were transformed with either the *Arabidopsis* Ohio cDNA library or the Clontech cDNA library in which the GAL4 activation domain was fused to *Arabidopsis* cDNAs. 100 μg of the Ohio cDNA library and 150 μg of the Clontech cDNA library were individually used for the transformations. To determine the transformation efficiencies, aliquots of the transformed cells were grown on the SD selective medium without tryptophan and leucine for selection of co-transformants. To identify the interacting proteins of DBD-AtHB6dC269, SD medium lacking tryptophan, leucine and histidine and containing 8 mM 3-AT were used for selection of *His3*-positive colonies. The Ohio library and Clontech library transformations yielded transformation efficiencies (number of colonies/ μg library DNA) of 6.5×10^4 and 1.68×10^4, respectively. Corresponding to the transformation efficiencies, 6.5×10^6 and 2.5×10^6 transformants were obtained, respectively. From the Ohio library transformants, 293 *His3*-positive colonies were obtained. The colony-lift filter assay demonstrated that 34 of these *His3* positives exhibited *LacZ*-positive reaction. From the Clontech library screening, 9 weak *His3*-positives were obtained, and only one of them was weakly *LacZ*-positive (Table I-9).

When AtHB6dC217 was used as the bait to screen the Ohio library, transformation with 100 μg plasmids of the Ohio cDNA library yielded a total of $1,05 \times 10^7$ transformants. From these transformants seventy-four *His3*-positive colonies were identified, and the colony-lift assay for *LacZ* expression resulted in only thirteen *LacZ*-positives (Table I-9).

In the screening of the Ohio library using the bait DBD-AtHB6dC119, 99 *His3* positive colonies were found from a pool of 3.8×10^6 transformants which were generated from transformation with 100 μg DNA. However, none of these *His3*-positive colonies were *LacZ*-positive (Table I-9).

Candidates that exhibited both *His3*- and *LacZ*-positive phenotype were selected as the candidates.

In order to selectively rescue the AD/prey plasmids the yeast plasmids were isolated from the positive candidates and were transformed into *E. coli* HB101. The HB101 strain carries the *LeuB* mutation and can be used to select for yeast plasmids bearing the *Leu2* marker (Boliver and Backman, 1979). The AD-plasmids bearing the *Leu2* marker (AD-preys) were isolated and then re-transformed into yeast harboring the fusion construct pGBT9-AtHB6dC269 to eliminate false positive transformants. 26 out of the 34 candidates from the Ohio library screened in the presence of AtHB6dC269 and 13 candidates from the Ohio library screened with AtHB6dC217 were successfully analyzed in that way and displayed both *His3- and LacZ*-positive, indicating the identification of true interacting partners of AtHB6.

Table I-9. Numbers of candidates screened from *Arabidopsis* cDNA library by different AtHB6 versions

Bait (BD-fusions)	Transformants screened	*His3*^{+} candidates	*His3*^{+} and *LacZ*[+] candidates	*His3*^{+}and *LacZ*[+] candidates after re-transformation
AtHB6dC269	$6,5 \times 10^6$ (Ohio lib.)	293	34	26
AtHB6dC269	2.5×10^6 (Clontech lib.)	9	1	0
AtHB6dC217	$1,0 \times 10^7$ (Ohio lib.)	74	13	13
AtHB6dC119	$3,8 \times 10^6$ (Ohio lib.)	99	0	0

Classification of the candidates

To eliminate duplicated colonies bearing the identical AD/library plasmid, the AD/library inserts of all candidates screened from the Ohio library (including those candidates obtained no re-transformants) were amplified by PCR using AD-forward and reverse primers. PCR products were then digested by *Alu*I, *Hind*III, *EcoR*I plus *EcoR*V, or *Nco*I plus *Xba*I, respectively. The restriction digestion patterns for each PCR product of the inserts were then compared with each other. According to the lengths of PCR fragments and restriction digestion patterns, candidates were divided into 3 classes (Figure I-13). Among the 34 candidates screened from Ohio library by AtHB6dC269, 24 exhibited the same pattern and were grouped into class A. Eight gave a second pattern

and were grouped into class B. The remaining two candidates formed the class C. The 13 candidates screened by AtHB6dC217 showed the same restriction digestion patterns as those candidates in class A therefore were grouped into class A. The only one candidate screened from Clontech library, which was not checked by restriction digestion, was classified in another Class D (Table I-10).

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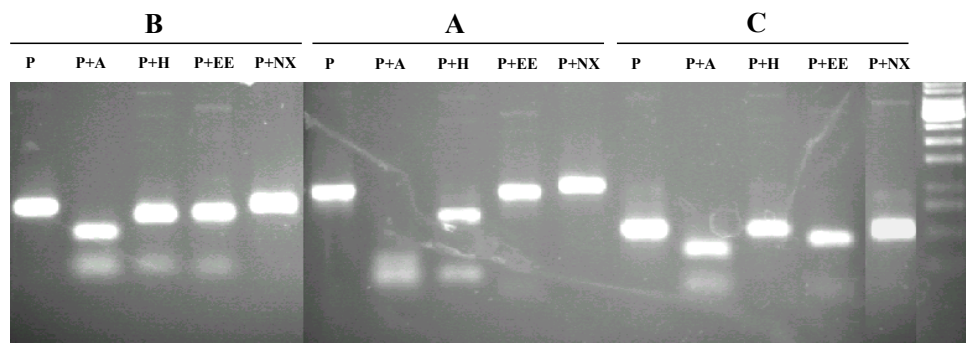
Figure I-13. The finger printing of candidates grouped into 3 different classes.   P: PCR product, P+A: PCR product digested by *Alu*I, P+H: PCR product digested by *Hind*III, P+EE: PCR product digested by *EcoR*I and *EcoR*V, P+NX: PCR product digested by *Nco*I and *Xba*I.

Table I-10. Classification of candidates identified from *Arabidopsis* cDNA library by using different AtHB6 versions.

Bait (DBD-fusions)	Positive candidates	Class			
		A	**B**	**C**	**D**
AtHB6dC269	34	24	8	2	0
AtHB6dC269	1	0	0	0	1
AtHB6dC217	13	13	0	0	0
AtHB6dC119	0	0	0	0	0

3.1.3 AtHB7 and AtGluRS ---- the new interaction partners of AtHB6

Sequence comparison in database

The PCR DNA fragments generated by PCR amplification of the cDNA of one to two candidates from each class were sequenced. Sequence analysis by BLAST (Altschul *et al.*, 1997) revealed that candidates in class A were identical to the cDNA of *Arabidopsis*

thaliana Glutamyl-tRNA synthetase (AtGluRS) (accession number AF067773), coding for the N-terminal portion (-261aa) of the AtGluRS protein with an additional 20 amino acids appended to its N-terminal (designated as AtGluRS(-20-261)). The 20 amino acids were encoded by 60 nucleotides upstream of the open reading frame. Candidates in class B were identical to the complement *Arabidopsis thaliana* mRNA AtHB7 (accession number X67032.1), coding for the N-terminal part (-136 amino acids) of AtHB7 with an additional 16 amino acids appended to the N-terminal (designated as AtHB7dC). These 16 amino acids were encoded by additional 48 nucleotides upstream of the open reading frame. Candidates in class C were identical to the *Arabidopsis thaliana* 18s RNA gene (accession X16077.1) and inserted in a false direction to AD, it did not code for any protein. Candidate D screened from Clontech library was identical to an *Arabidopsis thaliana* "unknown protein" mRNA (accession AF370137), but the reading frame was shifted when compared with the open reading frame of GAL4-AD. It coded for a short peptide of 33 amino acids with no identity to any known proteins. Thus, from the screening efforts, only two partners AtGluRS(-20-261) and AtHB7dC interacting with AtHB6dC269 were identified. The database sequence information of the candidates is briefly summarized in Table I-11 and the nucleotide and amino acid sequences of the candidates are shown in Appendix 3.

Table I-11. Databank comparison of the insert sequences of the candidates screened by pGBT9-HB6dC269

Candidates	Identical sequences in databank		Identical protein sequences /total length)	Coding protein domain of DBD fusion
	Accession number	Gene		
Class A: AII-2, AIII-63	AF067773	Glutamyl-tRNA synthetase (At5g26707)	AtGluRS (719aa)	-20~261 aa of AtGluRS
Class B: AV-130	X67032.1	AtHB7 (At2g46680)	AtHB7 (258aa)	-16~136 aa of AtHB7
Class C: AV-72	X16077.1	18s rRNA (Candidate corresponding to the nucleotides of 1665-~1328)	no	no
D1	AF370137	Unknown (At3g04830) protein, predicted 299aa	no	33aa

Dimerization of AtHB5, AtHB6 and AtHB7 in yeast

The homeodomain protein AtHB5 was reported to be able to form homodimers and

heterodimers *in vitro* with other classI HD-ZIP proteins such as AtHB6, AtHB7, AtHB12 (Johannesson *et al.*, 2001), and the dimerization of homeodomain proteins could be required to perform their function on gene regulation through specific DNA binding (Sessa *et al.*, 1997). In my experiment, AtHB7 was identified as an interaction partner of AtHB6 in the yeast two-hybrid system, suggesting the formation of a heterodimer between AtHB6 and AtHB7. To demonstrate the potential of dimerization between the related HD-ZIP proteins, a two-hybrid interaction analysis with AtHB6, AtHB7 and AtHB5 was performed in more detail.

AtHB6-AtHB6:

To test for the possibility of homodimerization of AtHB6, the cDNA fragments coding for AtHB6dC217, AtHB6dC269 and AtHB6dC119 were generated from the corresponding pGBT9- constructs and cloned into pGAD424. The schematic diagram of pGAD424 with the different AtHB6 versions is shown in Figure I-14.
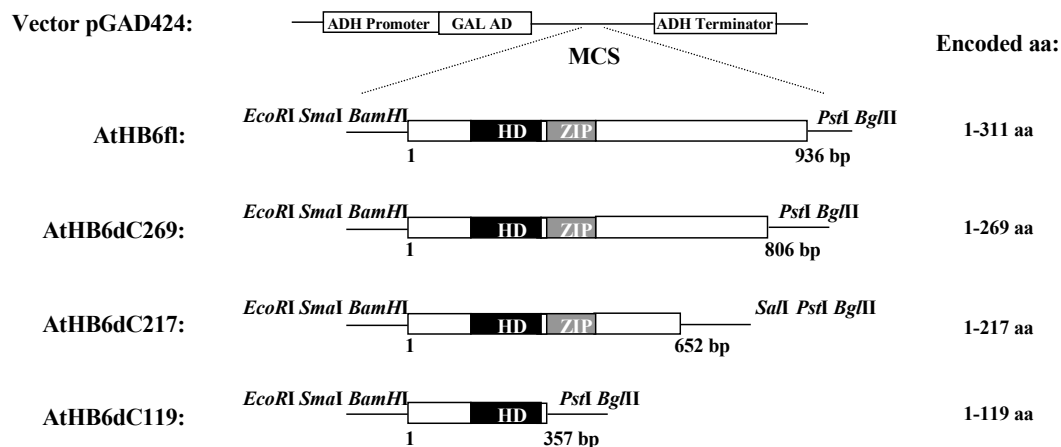
Figure I-14. Schematic diagram of the constructs of different AtHB6 versions in pGAD424. Regions coding for homeodomain (HD) and leucine zipper (ZIP) in AtHB6, and the multiple cloning sites in the pGAD424 vector are presented. The nucleotide length as well as the numbers of aa encoded by the cDNA constructs of AtHB6 is given.

The yeast HF7c harboring pGBT9-HB6dC269 was transformed with pGAD424-HB6fl, pGAD424- HB6dC119, pGAD424- HB6dC217, pGAD424- HB6dC269 or pGAD424. β-galactosidase activity in transformants was analyzed by the liquid culture assay using ONPG as substrate to determine the activation of the *LacZ* reporter gene. The results indicated that all combinations had very low β-galactosidase activity (Figure I-15), suggesting that there was no interaction between AtHB6dC269 and the different versions of AtHB6. This implied either that no homodimers of AtHB6 could be formed

in this system, or that the truncated version AtHB6dC269 is not sufficient to form homodimers with AtHB6. Unfortunately, it is impossible to perceive the interaction between two the full-length AtHB6 proteins in this system because the full-length AtHB6 fused to DNA-binding domain can activate the reporter genes autonomously.
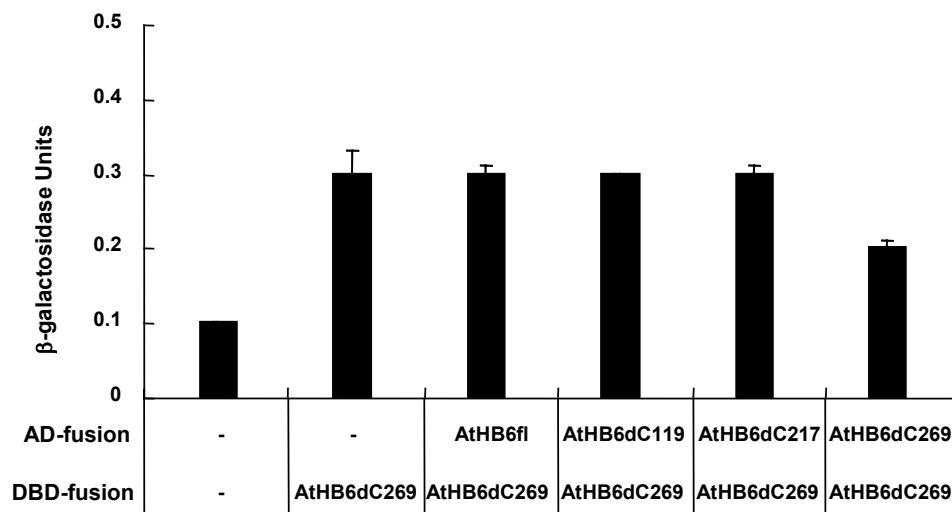
Figure I-15. Quantitation of the interaction between AtHB6dC269 fused to the *GAL4* DNA binding domain (DBD) and different AtHB6 versions fused to the *GAL4* activation domain (AD) in the yeast two-hybrid system by β-galactosidase liquid assay. 1 unit of β-galactosidase is defined as the amount of enzyme that hydrolyzes 1 μmol of ONPG to o-nitrophenol and D-galactose per minute per cell. The data is presented as the average (±SD) of independent analyses of three single colony.

AtHB6-AtHB7:

AtHB7dC is an interacting partner of AtHB6dC269 and represents the N-terminal part of AtHB7 containing the homeodomain and leucine zipper (Figure I-16). It was fused to the AD in the pACT2 vector. In order to define the region of AtHB6 required for the interaction with AtHB7dC, interactions between AD-AtHB7dC and different versions of AtHB6 fused to DBD were tested using the yeast two-hybrid system. Yeast strain HF7c was co-transformed with a pACT2-HB7dC and pGBT9-HB6dC217, pGBT9-HB6dC269 or pGBT9-HB6dC119. Co-transformant [pACT2/ pGBT9] was used as the negative control. The β-galactosidase activity assay demonstrated (Figure I-17) that the *LacZ* reporter gene was activated by the combination of AD-AtHB7dC and DBD-AtHB6dC217 as well as by the combination of AD-AtHB7dC and DBD-AtHB6dC269. The β-galactosidase activity in the later combination was higher

than three times that of the former. The combination of DBD-AtHB6dC119 and AD-AtHB7dC exhibited only background levels of β-galactosidase activity. This result indicates that the homeodomain region in AtHB6 is not sufficient for interaction with AtHB7, the leucine zipper and even the C-terminal region immediately linked to the leucine zipper are required for this interaction.
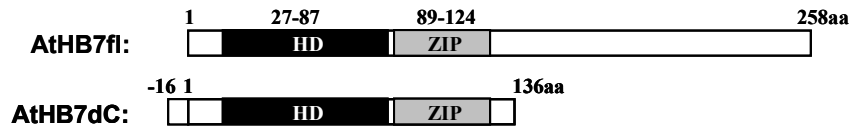
Figure I-16. Schematic of different AtHB7 versions. The homeodomain and leucine zipper are presented by the dark and grey boxes, respectively. The '–16 to 1' amino acids are the 16 amino acids coded by the cDNA upstream of its open reading frame.
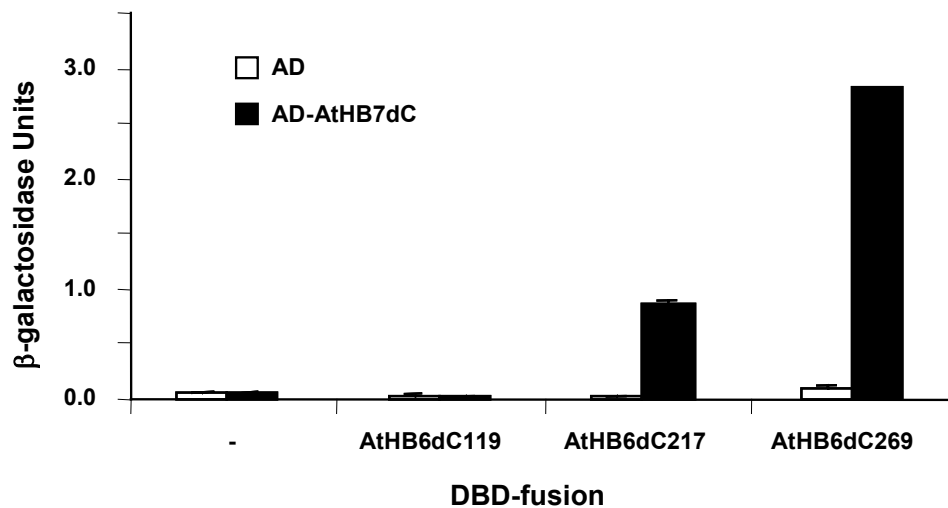
Figure I-17. Quantitation of two-hybrid interactions of AD-AtHB7dC with different AtHB6 versions fused to DBD using a β-galactosidase assay. The β-galactosidase activity is presented as the average (±SD) from three independent analyses.

The full-length of AtHB7 was further tested for potential interaction with AtHB6. Yeast strain HF7c was co-transformed with pGAD424-HB7fl and pGBT9 vectors expressing DBD fused with different versions of AtHB6. The β-galactosidase activity assay revealed that only the combination of AD-AtHB7fl and DBD-AtHB6dC269 could activate the reporter gene weakly (< 1.0 units). The combination of AD-AtHB7fl and

shorter versions of AtHB6 (AtHB6dC217, AtHB6dC119) fused to DBD gave only background levels of activity (Figure I-18). It further confirmed that the interaction of AtHB6 and AtHB7 requires not only the homeodomain and leucine zipper of AtHB6, but also the nearby C-terminal portion. Compared with AtHB7dC (in pACT2), the full-length AtHB7fl (in pGAD424) exhibited a weaker interaction with AtHB6 than the short version AtHB7dC did (Figure I-17, Figure I-18). It is possible that the C-terminal of AtHB7 negatively interfered with the interaction with AtHB6, or that AtHB7fl expressed in the vector pGAD424 with a low expression level (Clontech hand book) results in a reduced interaction.

AtHB6-AtHB5:

The interaction between AtHB6 and AtHB5 was also tested in a two-hybrid yeast system, but no interaction was detected (Figure I-18).
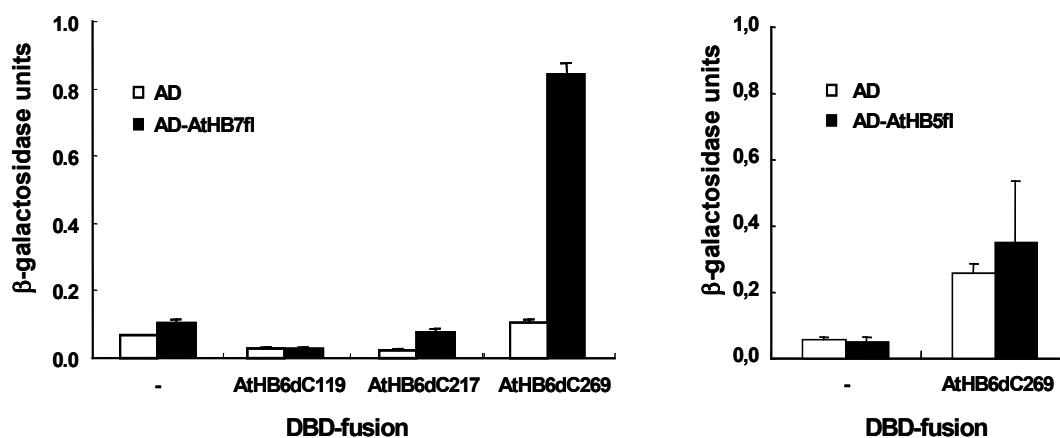
Figure I-18 Quantitation of two-hybrid interactions of AD-AtHB7fl and AD-AtHB5fl with different AtHB6 versions fused to DBD using a β-galactosidase assay. The β-galactosidase activity is presented as the average (±SD) from three independent analyses.

Protein domains critical for AtHB6-AtGluRS interaction

AtGluRS was identified as an interaction partner of AtHB6. In order to define the protein binding sites of AtGluRS with the concerned proteins, several AtGluRS versions were inserted into the pACT2 vector (Figure I-19A). These versions code for: the full-length AtGluRS (GluRSfl), truncations containing residues from 1-110

(AtGluRS1-110), 1-216 (AtGluRS1-216), 1-261 (AtGluRS1-261), 1-445 (AtGluRS1-445), 217-445 (AtGluRS217-445), 232-455 (AtGluRS232-455) and from 449-719 (AtGluRS449-719). All constructs were verified by DNA sequence analysis using the AD-forward primer and AD-reverse primer, and for the long inserts, using two additional primers GTS217f and GTS449f. Furthermore, expression of the proteins in yeast was tested by immuno-detection of the western blots of protein extracts using anti-AD antibody. All the tested constructs expressed the AD-fusions of predicted molecular mass, *e.g.*, AD is 17 kDa, AD-AtGluRS(1-110) is 29 kDa, AD-AtGluRS(1-216) is 40.8 kDa, AD-AtGluRS(1-261) is 45.6 kDa, AD-AtGluRS(217-445) is 42 kDa, AD-AtGluRS(449-719) is 46.7 kDa and AD-AtGluRS is 96 (Figure I-19). The results demonstrated that all constructs were correct cloning.
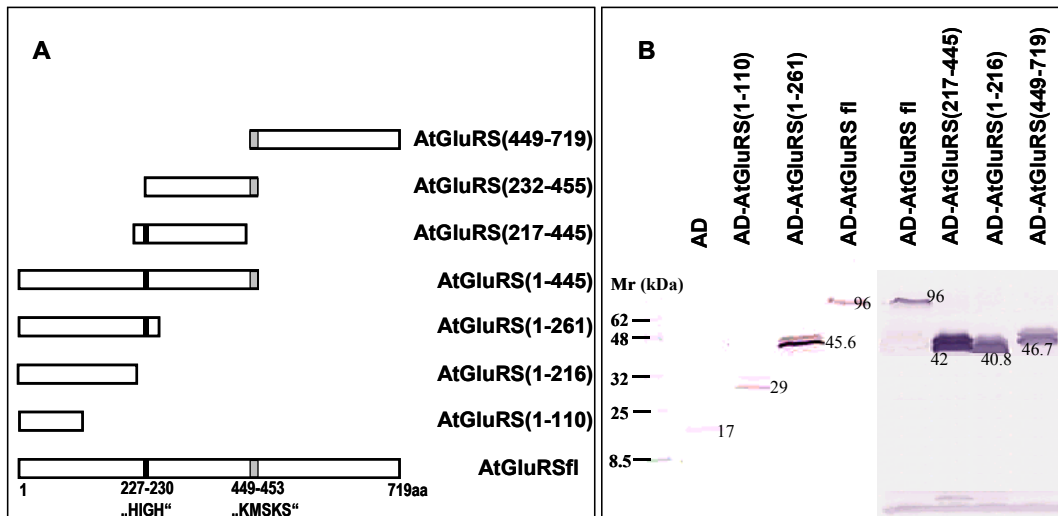
Figure I-19. A: Different AtGluRS expression versions constructed in pACT2 as AD-fusions. The solid box represents the putative ATP binding motif "HIGH", the hatch box represents the putative binding site "KMSKS" of the 3' end of the tRNA. B: Protein expression of pACT2-GluRS in yeast cells. Proteins were immuno-detected using mouse anti-AD and rabbit anti-mouse IgG-alkaline phosphatase antibodies.

To quantify the strength of interaction between AtGluRS(-20-261) and AtHB6, and to identify the region in AtHB6 required for the interaction, the yeast cells were co-transformed with pACT2-GluRS(-20-261) and pGBT9-HB6dC269, or pGBT9-HB6dC217, or pGBT9-HB6dC119. β-galactosidase activity of the co-transformants was assayed. Figure I-20 showed that the enzyme activity of co-transformants of pGBT9-HB6dC269 and pACT2-GluRS(-20-261) was very high with approximately 13 units. With carboxy terminal decrease in length of the

DBD-AtHB6 fusions, enzyme activity was concomitantly reduced. The β-galactosidase activity of co-transformants of pGBT9-HB6dC119 and pACT2-GluRS(-20-261) was about 6 units. However, it was still rather high, suggesting that the homeodomain in AtHB6 efficiently interacts with AtGluRS in yeast.
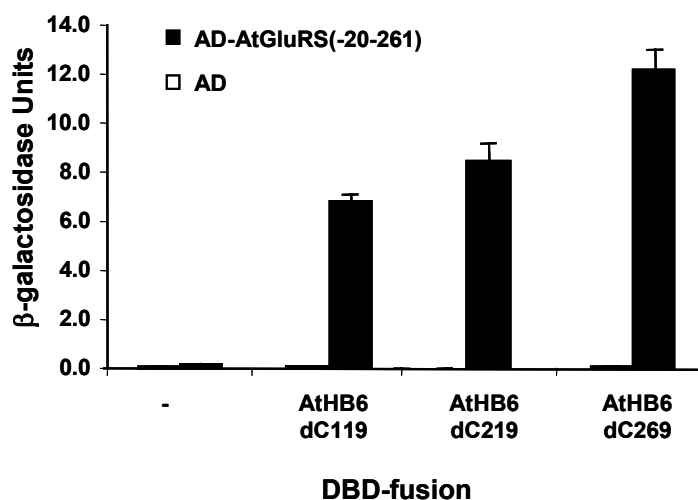
Figure I-20. Quantitative measurement of two-hybrid interactions of AtGluRS(-20-261) with different AtHB6 versions using a β-galactosidase assay. Yeast strain HF7c was co-transformed with pACT2-GluRS(-20-261) or pACT2 (control) as prey constructs, pGBT9-HB6dC219, pGBT9-HB6dC269, pGBT9-HB6dC119 or pGBT9 (control) as bait versions. The data is presented as the average (±SD) from three independent analyses.

Two-hybrid interaction assay with different versions of AtGluRS fused to AD and AtHB6dC269 fused to DBD was performed to identify the critical region in AtGluRS required for the interaction with AtHB6. The combination of DBD-AtHB6dC269 and AD-GluRS(1-261) could activate the *LacZ* reporter. DBD-AtHB6dC269 combined with other AD-AtGluRS fusions such as N-terminal AtGluRSs shorter or longer than AtGluRS(1-261), the C-terminal AtGluRS, and even the full-length AtGluRS, gave only background β-galactosidase activity (Figure I-21). Thus the N-terminal region of AtGluRS (1-261amino acids) is important for the interaction, while full-length AtGluRS cannot interact with AtHB6 in yeast. This was an unexpected result.
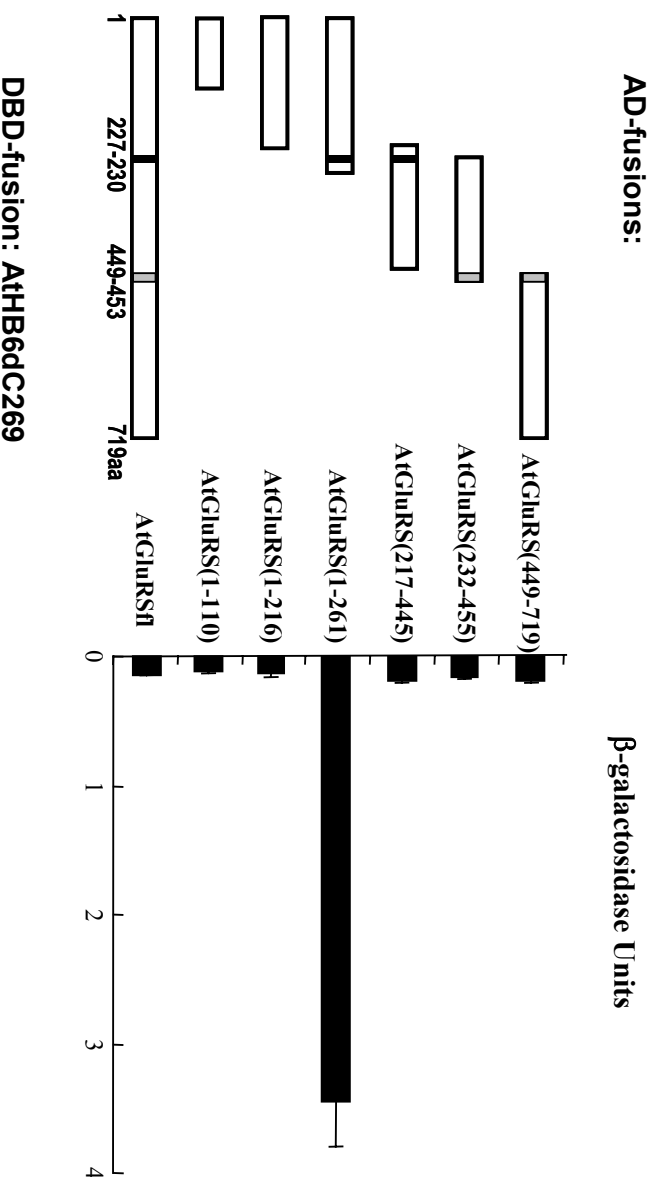
Figure I-21. Quantitative measurement of two-hybrid interactions of AtHB6dC269 with different AtGluRS versions using the β-galactosidase assay. The AD-GluRS versions are showing on the left, the dark box represents the putative ATP binding motif „HIGH", the grey box represents the putative binding site „KMSKS" of the 3' end of the tRNA.   Yeast strain HF7c was co-transformed with   pACT2-GluRSfl,   pACT2-GluRS(1-110),   pACT2-GluRS(1-261),   pACT2-GluRS(1-216),   pACT2-GluRS(217-445), pACT2-GluRS(232-455), pACT2-GluRS(449-719) or pACT2-GluRS(1-445) as prey and pGBT9-HB6dC269 as bait. The data is presented as the average (±SD) from three independent analyses.

To clarify the specificity of the interaction between *Arabidopsis* homeodomain proteins and AtGluRS, the cDNA fragment coding for amino acids 1-136 of AtHB7 (AtHB7dC136) was cloned to pGBT9 (Figure I-22), and this plasmid was then co-transformed to yeast with the pACT2 vector containing different versions of AtGluRS. The β-galactosidase activity assay (Figure I-23) revealed that the combination of DBD-AtHB7dC136 and AD-AtGluRS(1-261) could activate the *LacZ* gene, suggesting the AtGluRS interacts with homeodomain proteins and might be that the
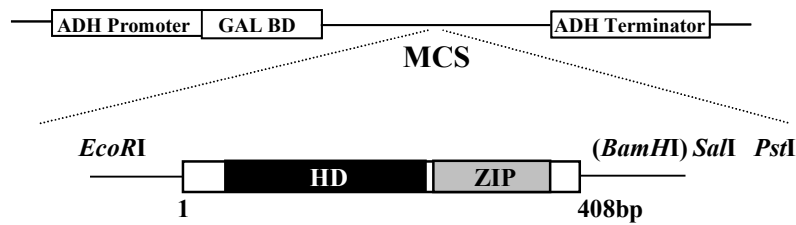
Figure I-22. Schematic diagram of pGBT9-HB7dC136. Regions coding for homeodomain (HD) and leucine zipper (ZIP) in AtHB7 as well as the multiple cloning sites in the pGBT9 vector are presented.
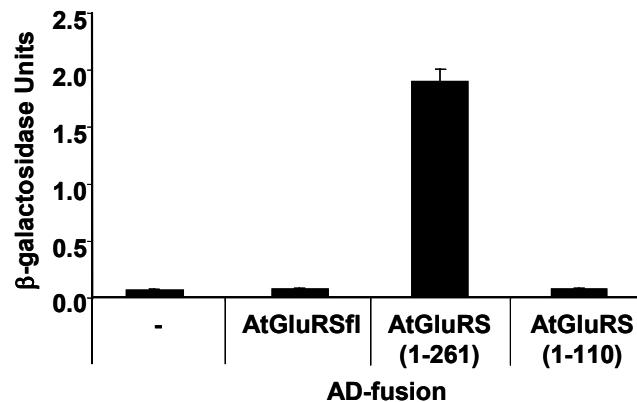
Figure I-23. Quantitative measurement of two-hybrid interactions of AtHB7dC136 with AtGluRS using a β-galactosidase assay. Yeast strain HF7c was co-transformed with pACT2-GluRSfl or pACT2-GluRS(1~261), or pACT2-GluRS(1~110), or the control pACT2 as prey and pGBT-HB7dC136 or the control pGBT9 as bait. The data is presented as the average (±SD) from three independent analyses.
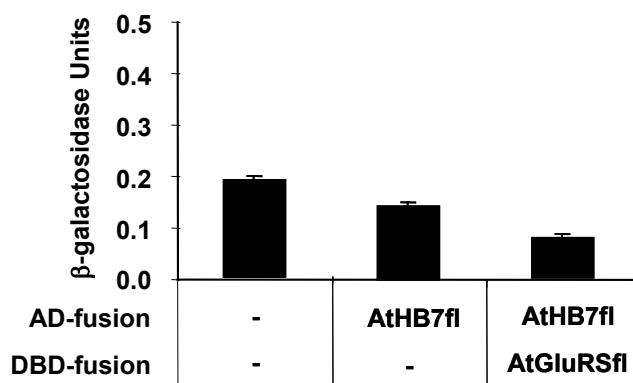
Figure I-24. β-Galactosidase assay of yeast cells co-tansformed with pGAD-HB7fl and pGBT-GluRSfl or empty vectors. The data is presented as the average (±SD) from three independent analyses.

interaction influence homeodomain protein-regulated gene transcription. However; the combination of DBD-AtHB7dC136 and AD-GluRSfl or the short version AD-GluRS(1-110) was not able to activate the reporter gene. And no activation of the *LacZ* gene was observed with pGAD424-HB7fl and pGBT9-GluRSfl co-transformed into yeast cells (Figure I-24). Results suggested the full-length AtGluRS somehow has no ability of interacting with other proteins in yeast.

3.2 Analysis of the interactions between AtHB6, PP2Cs and AtGluRS

AtHB6 interacts with ABI1 (Himmelbach et al., 2002), and AtGluRS is an interaction partner of ABI2 (Yang, 2003). Interestingly, the AtGluRS was identified as an interaction partner of AtHB6 in the screening performed in the present report. Thus a inter-relationship among AtHB6, AtGluRS and ABI1/ ABI2 is implied. Are there protein complexes formed, or are there some competitive interactions occurring between them? To clarify these questions, the following experiments were conducted.

3.2.1 Two-hybrid interaction of AtHB6 and ABI1/ABI2

Protein domains critical for AtHB6-ABI1 interaction

The interaction of AtHB6 and ABI1 has been reported by Himmelbach *et al.* (2002) by using ABI1dN (ABI1dN^{Gly180Asp}, amino acids 122-434), mutant type abi1dN and NAP (ABI1dN ^{Asp177Ala}, amino acids 122-434) as baits, and AtHB6, S67A (AtHB6 ^{Ser67Ala}), Δ1 (AtHB6 44-311aa) and Δ2 (AtHB6 44-217aa) as prey in a yeast two-hybrid assay. It demonstrated that the interaction of AtHB6 and ABI1 was dependent on the functional

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catalytic domain of ABI1 as well as the homeodomain of AtHB6. Here a new ABI1 version ABI1 Sac (amino acids 1-268) fused to DBD and AtHB6dC269 fused to AD were further included in the yeast two hybrid-interaction assay.

Figure I-25 demonstrates that the combination of AD-AtHB6fl and DBD-ABI1dN activated the expression of the reporter gene *LacZ* to a quite high level at 2 units. The combination of AD-AtHB6fl and DBD-abi1dN decreased the reporter gene expression to about one fourth of the former's. The combination of AD-AtHB6fl and DBD-NAP or DBD-ABI1 Sac only resulted in background reporter expression. These results indicate that not only the functional catalytic domain but also the C-terminal region (268-434aa) of ABI1 are required for interaction with AtHB6.

Compared with AtHB6fl, the C-terminal truncation of AtHB6 (AtHB6dC269) reduced the strength of interaction with ABI1 to a very low level (Figure I-25), suggesting that the C-terminal of AtHB6 is important for the interaction.
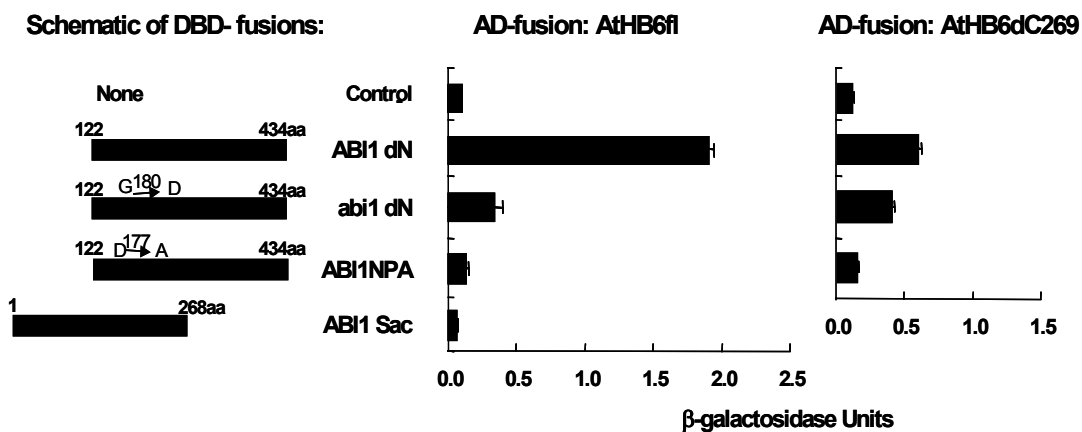
Figure I-25. Quantitative measurement of two-hybrid interactions of AtHB6 with ABI1 using a β-galactosidase assay. Yeast strain HF7c was co-transformed with a pGAD424-HB6fl or pGAD424-HB6dC269 and pGBT9-ABI1dN, pGBT9-abi1dN, pGBT9-ABI1NPA, pGBT9-ABI1 Sac, or the control pGBT9. The data is presented as the average (±SD) from three independent analyses.

Interaction between AtHB6 and ABI2

ABI2, another PP2C, which together with PP2C ABI1 controls ABA signal transduction in plants (Himmelbach *et al.*, 1998; Leung and Giraudat, 1998), was also assayed for interaction with HD-ZIP proteins in the yeast two-hybrid system.

The combination of DBD-ABI2 and AD-AtHB6dC269 was able to activate the reporter gene slightly, while the combination of AD-AtHB6dC269 and the mutant

DBD-abi2 (ABI2^{Gly168Asp}) exhibited no activation of the reporter gene (Figure I-26). This indicates that the catalytic domain of ABI2 as reported for ABI1 (Himmelbach *et al.*, 2002) is required for interaction with AtHB6.

When AD-AtHB5fl or AD-AtHB7fl is combined with DBD-ABI2 or DBD-abi2 in the yeast two-hybrid system, only background levels of β-galactosidase activity were detected (Figure I-26). Thus no detectable interaction occurred between AtHB5 or AtHB7 and ABI2.

3.2.2 Two-hybrid interaction of AtGluRS and ABI1/ABI2

Previous results have shown that interactions occur between AtHB6 and ABI1 or ABI2, and between AtGluRS and AtHB6. AtGluRS was also identified as an interaction partner of ABI2 (Yang, 2003). The interaction between AtGluRS and ABI1 or ABI2 respectively, was further examined.

Protein domains critical for ABI1-AtGluRS interaction

Yang (2003) demonstrated that the phosphatase activity of ABI1 was not critical for the interaction of AtGluRS and ABI1 when using yeast two-hybrid system to detect the interaction of AtGluRS(-20-261) and ABI1dN or abi1dN and NPA. To identify contact sites on ABI1 interacting with AtGluRS, a carboxy terminal version of ABI1 ABI1dN262 which excludes the phosphatase catalytic domain was generated as a DBD-fusion protein. The constructs pGBT9-ABI1dN262 as well as the other ABI1 constructs (Figure I-27) were co-transformed with pACT2-GluRS(-20-261) into yeast, and the activation of the reporter gene *LacZ* was determined. The wild type C-terminal ABI1dN, the protein phosphatase deficient mutant abi1dN, and even the non-active protein phosphatase ABI1NAP strongly activated the expression of the reporter gene. The C-terminal short version ABI1dN262 activated the expression of *LacZ* at a residual level of around 18% compared with ABI1dN. However, it was still strong. While the C-terminal truncation (ABI1 Sac) completely abolished the reporter gene's activation to a background level of 3% (Figure I-27). These results imply that the interaction between AtGluRS and ABI1 is not dependent on the phosphatase activity of ABI1, and the C-terminal 262-434 aa of ABI1 are major determinants for the interaction with AtGluRS.

To identify the critical regions required for the interaction with ABI1, different AtGluRS versions inserted in pACT2 were combined with ABI1dN inserted in pGBT9 and the activation of *LacZ* gene in the co-tansformants was determined. The β-galactosidase activity could be detected only in the combination of AD-AtGluRS
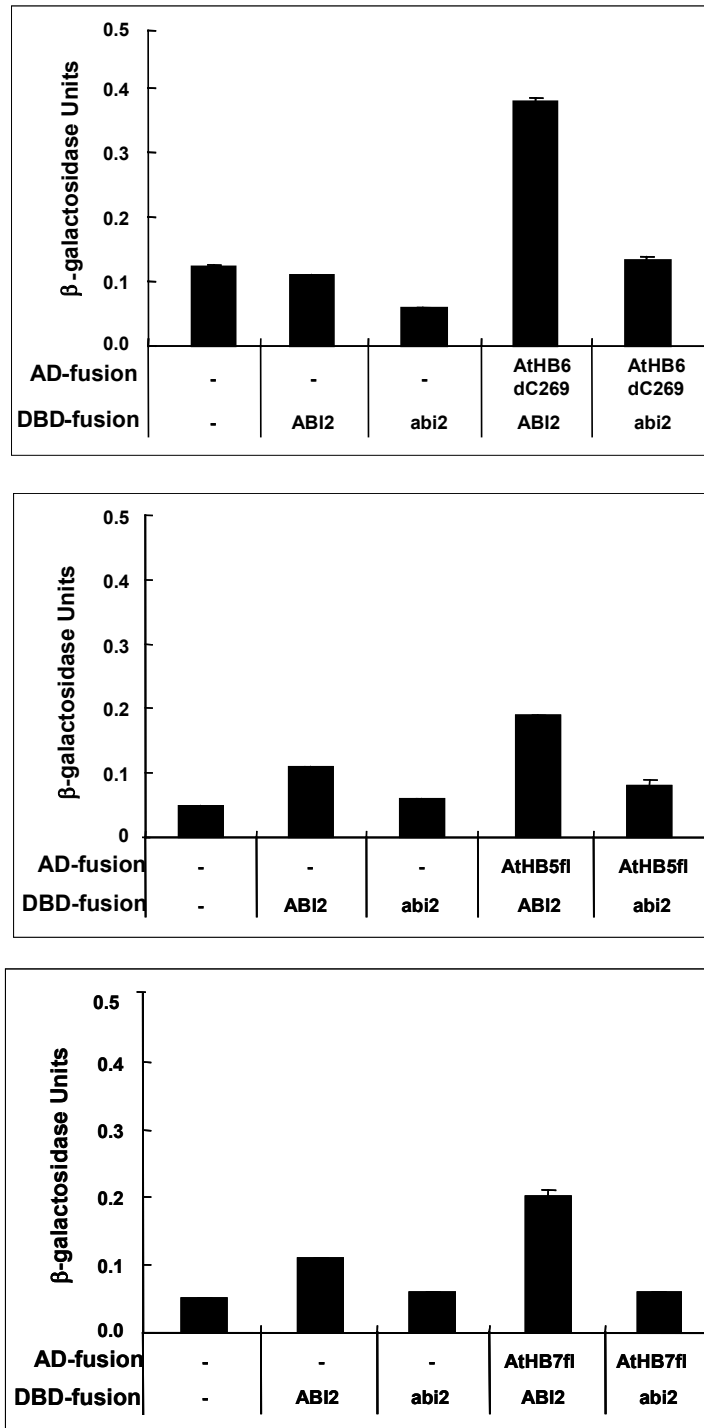
Figure I-26. Quantitative measurement of two-hybrid interactions between HD-ZIP proteins and ABI2 using β-galactosidase activity for assaying interaction. The AD-fusions were expressed in pGAD424 vector, DBD-fusions were expressed in pGBT9 vector. The data is presented as the average (±SD) from three independent analyses.

DBD- fusions: **β-galactosidase Units**

None   Control

262   434aa   ABI1dN262

1   268aa   ABI1 Sac

122 D→177A   434aa   ABI1NPA

122 G→180D   434aa   abi1 dN

122   434aa   ABI1dN

0   5   10   15   20

AD-fusion: AtGluRS (-20-261)
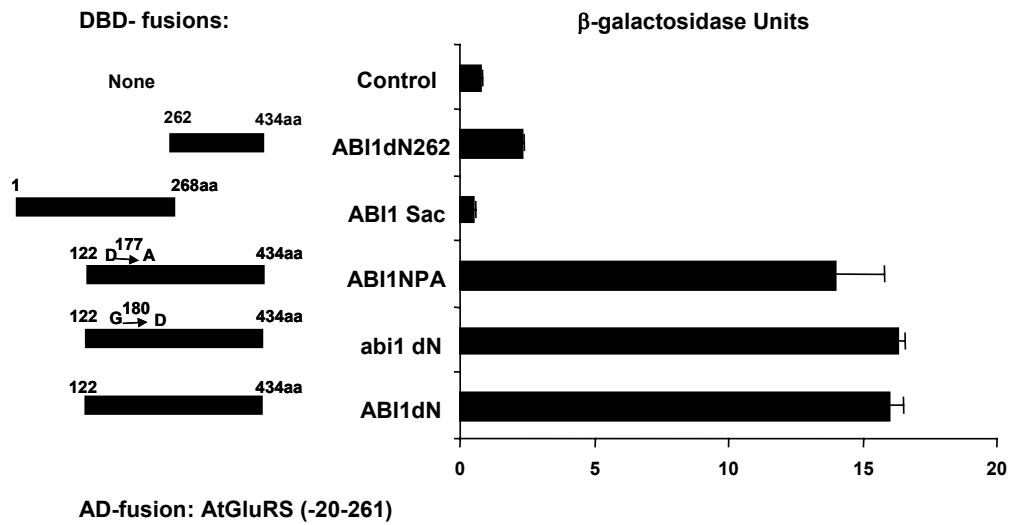
Figure I-27. Quantitative measurement of two-hybrid interactions of AtGluRS(-20-261) with different ABI1 versions using a β-galactosidase assay. Yeast strain HF7c was co-transformed with a pACT2-GluRS(-20-261) prey (pACT2 as the control) and either pGBT-ABI1dN, pGBT-abi1dN, pGBT-ABI1dND60A, pGBT-ABI1Sac, pGBT-ABI1dN262 or empty pGBT9 as bait. The data is presented as the average (±SD) from three independent analyses.

AD- fusions: **β -galactosidase Units**

„HIGH"   „KMSKS"
1   227-230 449-453   719aa   AtGluRSfl

AtGluRS(1-110)

AtGluRS(1-216)

AtGluRS(1-261)

AtGluRS(217-445)

AtGluRS(232-455)

AtGluRS(449-719)

0   1   2   3   4   5

DBD-fusion: ABI1dN
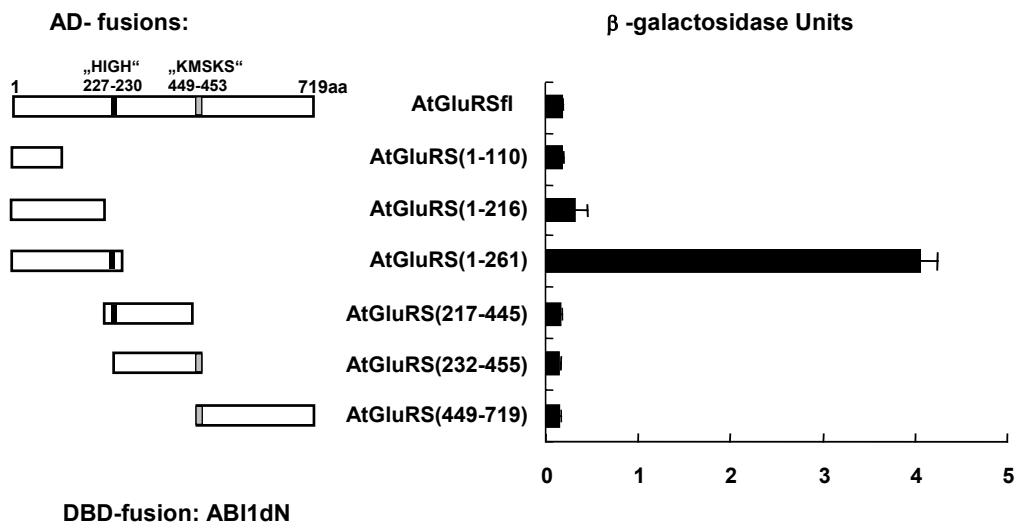
Figure I-28. Quantitative measurement of two-hybrid interactions of ABI1dN with different AtGluRS versions using a β-galactosidase assay. Yeast strain HF7c was co-transformed with pGBT-ABI1dN and pACT2 vector expressing different AD-AtGluRS versions as shown on the left. The data is presented as the average (±SD) from three independent colonies.

(1-261) and DBD-ABI1dN (Figure I-28). The combination of the other AtGluRS versions with DBD-ABI1dN failed to activate reporter gene expression. As the results of sequencing and protein expression (Figure I-19) showed that all constructs were correct cloning, no detectable interaction between AtGluRSfl and ABI1 might be explained as that the presence of C-terminal of AtGluRS interferes with the interaction.

Interaction between ABI2 and AtGluRS

The interaction of AtGluRS with ABI2 was tested in parellel. The data obtained in the two-hybrid system is showed in Figure I-29. It indicates that ABI2 interacted with AtGluRS(-20-261) in the yeast system, while no interaction was detected between the mutant abi2 and AtGluRS (-20-261). The functional catalytic domain of ABI2 most likely plays an important role in this interaction.

| AD -fusion | - | - | - | AtGluRS (-20-261) | AtGluRS (-20-261) |
| DBD-fusion | - | ABI2 | abi2 | ABI2 | abi2 |
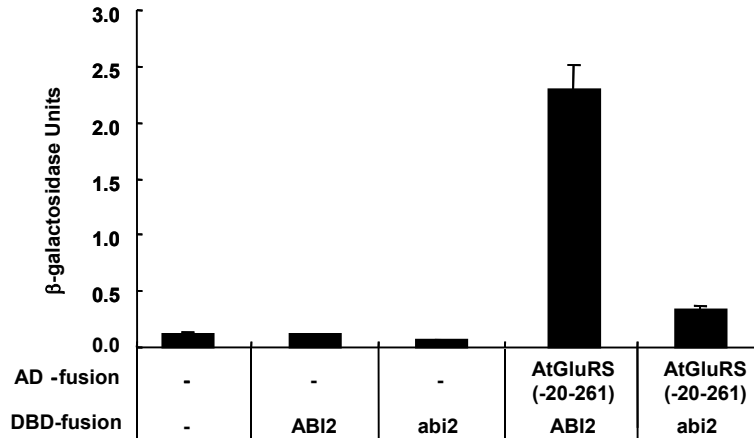
Figure I-29. Quantitative measurement of two-hybrid interactions of ABI2 or abi2 and GluRS(-20-261) using a β-galactosidase liquid assay. Yeast cells were co-transformed with pGBT-ABI2 or pGBT-abi2 as baits and pACT2-GluRS(-20-261) as prey. The data is presented as the average (±SD) from three independent analyses.

3.2.3 Physical interaction between ABI1/ abi1 and AtGluRS *in vitro*

A clear and strong interaction between ABI1 or abi1 and the deleted version of AtGluRS (AtGluRS(1-261)) was demonstrated by the yeast two-hybrid system. However, no interaction between the full-length AtGluRS and ABI1 or abi1 was detected in this system. Therefore the question arises whether there is no interaction indeed between the entire AtGluRS and ABI1/ abi1, or, whether there is interaction taking place but cannot be assayed by the yeast system, *e.g.* lack of proper folding or functioning of AtGluRS as

a fusion protein. In order to clarify this point, the physical interaction analysis between ABI1 or abi1 and AtGluRS was attempted *in vitro* with the assistance of affinity tagged proteins purified from *E. coli*.

Expression and purification of GST–AtGluRS fusion protein

An AtGluRS fusion glutathione S-transferase (GST) has been constructed in the pGEX vector Yang (2003) as illustrated in Figure I-30 by. The plasmid was transformed into *E. coli* DH5α for over-expression of the fusion protein.

   To optimize the conditions for fusion protein expression, small-scale cultures of the (pGEX-GluRS)-transformed *E. coli* were cultivated at two different temperatures (26 ^{o}C and 30 ^{o}C). To induce the expression of the fusion protein, 0, 0,5 and 1.0 mM IPTG were supplied to the bacterial culture when the OD_{600} of the cultures reached 0.6~0.8. After another 2 hrs incubation, the protein was extracted and western-blotting was performed. GST-GluRS was detected by mouse anti-GST antibody (α-GST) and anti-mouse IgG alkaline phosphatase conjugate (IgG-AP). Results revealed that low temperature (26 ^{o}C) reduced the background of low molecular mass protein with no effect on the expression of the GST-AtGluRS fusion protein of interest (Figure I-31). The concentration of IPTG was not critical. 0.2 to 1.0mM IPTG could induce the expression of GST-fusion protein with no difference.
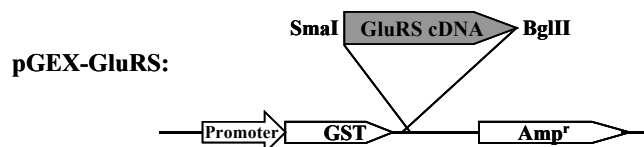
pGEX-GluRS:

Figure I-30. Schematic diagram of pGEX-GluRS. The coding region of AtGluRS cDNA was inserted into the *Sma*I – *Bgl*II sites of pGEX.

   To purify the GST-AtGluRS fusion protein, (pGEX-GluRS)-transformed *E. coli* were cultivated at 26 ^{o}C and the expression was induced by 0.2 mM IPTG. The protein extracted from these cells was purified by affinity-chromatography using a glutathione-Sepharose 4B column. Figure I-32 shows that the GST-AtGluRS protein was not detected in lysates of non-induced cells, while 0.2 mM IPTG efficiently induced the expression of GST-AtGluRS. For purification, total protein extracts were passed through a glutathione-Sepharose 4B column. The Sepharose was subsequently incubated with reduced glutathione to elute the bound protein. Most proteins were not bound on the column and present in the flow-through fraction; whereas the GST-AtGluRS was

recovered in the glutathione-elution fraction.

Affinity interaction assay of GST-AtGluRS and ABI1 /abi1

ABI1 and abi1 containing an N-terminal histidine tag were expressed from plasmid pQE70-*ABI1* or pQE70-*abi1* in *E. coli.* (Leube *et al.*, 1998) and were purified under native conditions using Ni-NTA resin.

   To perform the affinity interaction assay, ABI1 or abi1 were tethered on Ni-NTA resin by incubation in binding buffer [300 mM NaCl; 10 mM Mg-acetate; 30 mM Na-Phosphate buffer (pH 7.0); 5 mM β-Me]. Afterwards the GST-AtGluRS or GST as a control was added to allow association with ABI1 or abi1. Subsequently, the beads were pelleted and the supernatant was collected as the supernatant fraction (S). Finally proteins were eluted by incubation with elution buffer to obtain the eluted fraction (E). Western blots of comparable aliquots detected by anti-ABI1-GST showed that over 90% of GST-AtGluRS as well as the GST was recovered with ABI1 in the eluted fraction (Figure I-33). It was reasoned that unspecific binding prevailed under these conditions.

   There are several possibilities that might explain the binding of GST to the ABI1-Ni-NTA resin: (1) GST was tethered on the Ni-resin *e.g.* by histidine residues; (2) GST was bound through hydrophobic interactions; or (3) GST interacts specifically with ABI1. Hence, experiments were designed to find stringent conditions for the protein interaction in order to reduce the background. To ascertain this: (1) 30 mM imidazol included in the binding buffer was used to reduce the possible affinity binding of GST to Ni^{2+}; (2) The detergents Triton X-100 (0.5%) and SDS (0.05%) were added to the binding buffer to disrupt hydrophobic interactions. The results showed that the original binding buffer or the original buffer containing imidazol were unable to diminish the binding of GST to Ni-NTA resin, while Triton-X100 and SDS efficiently reduced the nonspecific binding (Figure I-34 I), indicating that the binding of GST on Ni-NTA resin was mainly due to hydrophobic interactions. The optimized binding- buffer [300 mM NaCl, 10 mM Mg-acetate, 30 mM Na-Phosphate buffer (pH7.0), 5 mM β-Me, 0.5% Triton-X and 0.05% SDS] was further used to test the possibility of the nonspecific binding of GST-GluRS to Ni-NTA-resin and the specific interaction between GST and ABI1. Figure I-34 II shows that GST-AtGluRS was present in the supernatant (S) not in the eluted fraction (E), showing that GST-AtGluRS is not able to bind under these conditions to the Ni-resin. In additional, GST was present in the supernatant while ABI1 was in the eluted fraction after the co-incubation of GST, ABI1 and Ni-NTA resin (Figure I-34 III). Hence, the immobilization of ABI1 on Ni-NTA resin was stable under these conditions and interaction between GST and ABI1 was low.

Temperature:	26 ºC				30 ºC			
IPTG Conc. (mM):	0	0.2	0.5	1.0	0	0.2	0.5	1.0
Ponceau S staining:								
WB: α-GST								

GST-AtGluRS (105 KDa)

Background (26 KDa)
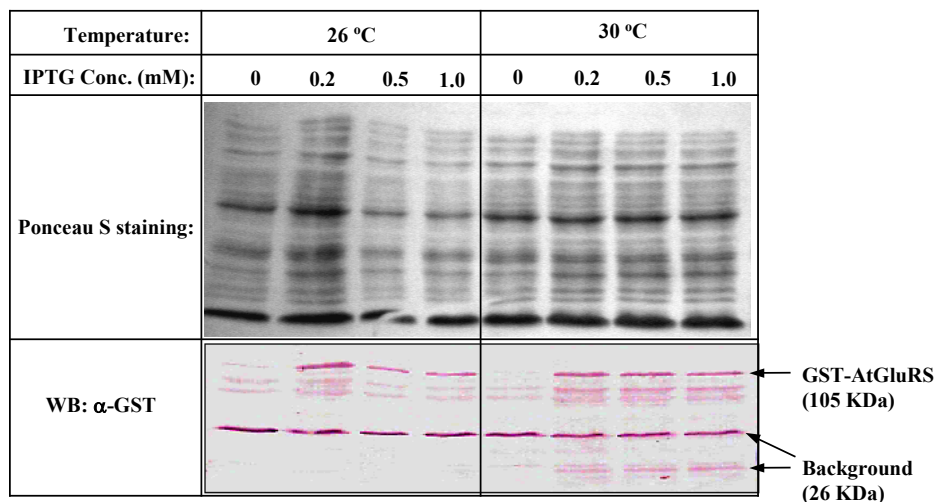
Figure I-31. Induction of GST-AtGluRS under different conditions. The cells were lysed directly by boiling in SDS-dye buffer and the supernatants were separated on 12% SDS-PAGE and transferred onto a nitrocellulose membrane. Amount of total protein was indicated by Ponceau S staining (top). Immuno-detection of western blots (WB) was performed using mouse anti-GST antibody (α-GST) and subsequently the secondary antibody goat anti-mouse IgG conjugated with alkaline phosphatase (bottom).

Silver staining   WB: α-GST

A   B   C   D   A   B   C   D

GST-AtGluRS

Background band
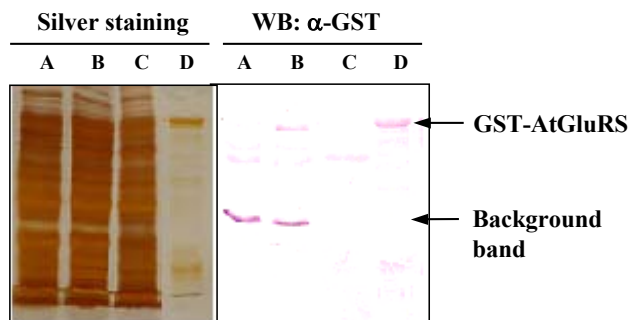
Figure I-32. Purification of fusion protein GST-AtGluRS. Protein extracts prepared from (pGEX-GluRS)-transformed *E. coli* cells were purified on a glutathione-Sepharose 4B column. Protein samples from total lysate of cells before addition of IPTG (A), protein samples from total lysate of cells induced by IPTG (B), flow-through (C), and sepharose affinity purified proteins eluted with 10 mM reduced glutathione (D) were separated on 12% SDS-PAGE for silver staining, or further transferred to a nitrocellulose membrane for immuno-detection with anti-GST antibody and the goat anti-mouse-alkaline phosphatase antibody.
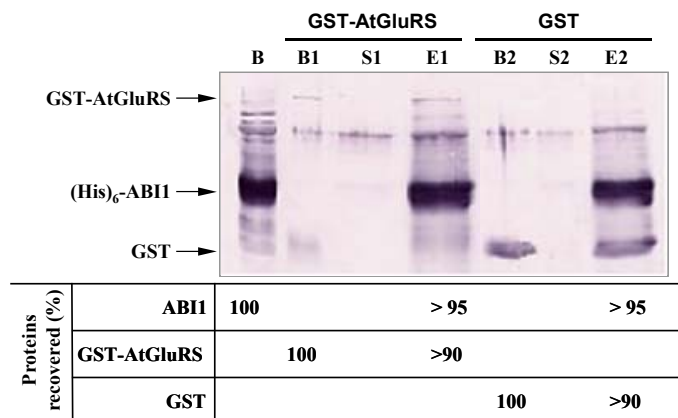
Figure I-33. Affinity interaction between GST-AtGluRS/GST and ABI1 *in vitro*. Comparable aliquotes of samples before incubation with nickel resin: B - ABI1, B1 - GST-AtGluRS, B2 – GST, supernatant after incubation with ABI1-Ni-resin: S1 - GST-AtGluRS, S2 – GST, as well as the elutes from the resin: E1 - GST-AtGluRS, E2 – GST, were separated on 12% SDS-PAGE. Western-blots on a nitrocellulose membrane were further immuno-detected with anti-ABI1-GST and the goat anti-mouse-alkaline phosphatase antibody.
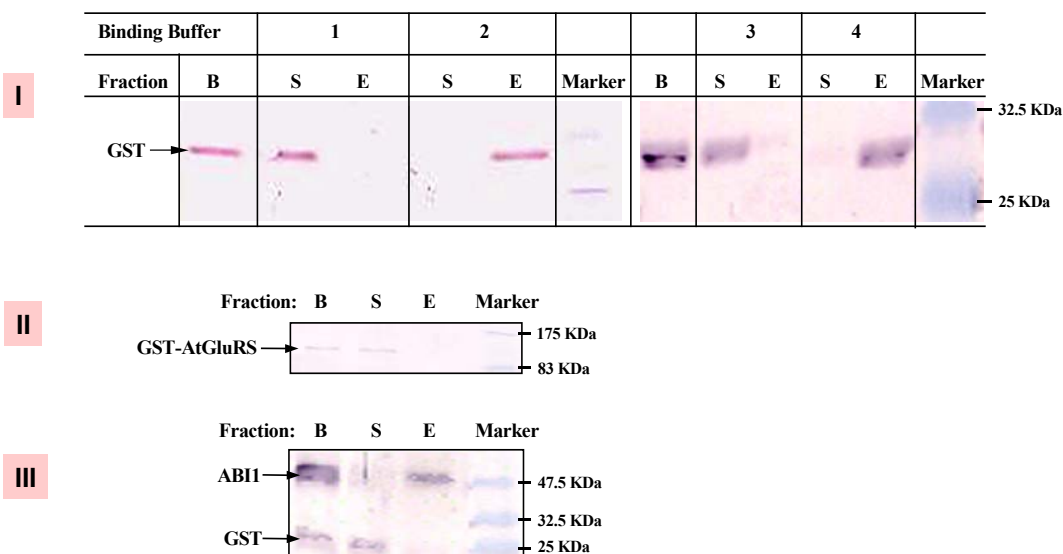
Figure I-34. Unspecific binding of GST or GST-AtGluRS to Ni-resin. GST or GST-AtGluRS was incubated with Ni-NTA resin in binding buffer for 40 min at 4 $^{\circ}$C and obtained the supernatant and resin pellet for elution. Comparable aliquots of the supernatant (S), elutes (E) and the original protein samples (B) were separated on 12% SDS-PAGE and blotted onto a nitrocellulose membrane for immuno-detection.
(I). The binding of GST on Ni-NTA resin at different stringent conditions of binding buffer with additional: 1. 0.5% Triton-X and 0.05% SDS; 2. 30 mM Imidazol; 3. 0.5% Triton-X, 0.05% SDS and 30 mM Imidazol; 4. None.
(II). The binding of GST-AtGluRS on Ni-NTA resin in optimized binding buffer cotaining 0.5% Triton-X and 0.05% SDS.
(III). The binding of GST on Ni-NTA-resin with immobilized ABI1 in that optimized binding buffer.

The next step was to determine if AtGluRS could associate with ABI1 and abi1. The GST or GST-AtGluRS was incubated with the ABI1 (or abi1)-immobilized on Ni-NTA resin. Western blot analysis of equal aliquots of samples and image analysis of signal density by "*MOLECULAR ANALYS*" demonstrates that more than 70% of the GST-AtGluRS was present in the eluted fraction and less than 30% was in the supernatant (Figure I-35). In the control with GST, only 35-40% was in the eluted fraction and more than 60% was in the supernatant.

These results suggest that AtGluRS interacts physically with both ABI1 and abi1.

	(His)$_6$-ABI1				(His)$_6$-*abi1*			
	B1	B2	S	E	B1	B2	S	E
ABI1/ *abi1*	100			100	100			100
GST-AtGluRS		100	30	70		100	29	71
GST		100	60	40		100	65	35
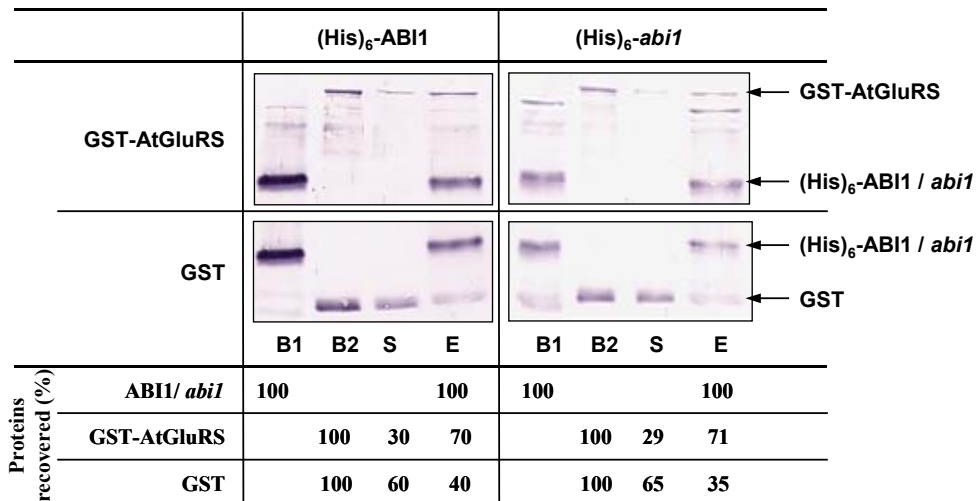
Figure I-35. Affinity interaction between GST-AtGluRS/GST and ABI1 in optimized binding buffer. Comparable aliquots of the sample of ABI1 (or abi1) before incubation with nickel resin (B1), the sample of GST-GluRS (or the control GST) before associating with ABI1 (abi1)-Ni-resin (B2), supernatant after associating with ABI1 (or abi1)-Ni-resin (S), and elutes from the resin (E) were separated on 12% SDS-PAGE and blotted onto a nitrocellulose membrane for immunodetection with anti-ABI1-GST and the goat anti-mouse-alkaline phosphatase antibodies.

3.2.4 Co-immunoprecipitation of AtGluRS and ABI1 from maize protoplasts *in vivo*

In order to corroborate the protein interaction *in planta*, AtGluRS and ABI1 were expressed in maize protoplasts. Physical interaction between these proteins was assayed by co-immunoprecipitation experiments using protein extracts and the corresponding antibodies.

The protoplast expression system has been used to study the ABA signal transduction mechanism (Sheen, 1996; 1998; Wu *et al.*, 1997). This system is a sensitive and fast

transient system to analyze gene expression. It experimentally yields large amounts of recombinant proteins, thus facilitating further immunological studies. In this study, the two immunoprecipitating partner proteins AtGluRS and ABI1 were expressed in maize protoplasts as fusion proteins. These fusions were generated by cloning the AtGluRS cDNA into pMENCHU to provide a HA (hemagglutinin) epitope tag at its C-terminus and by cloning the ABI1 cDNA into pMESHI thereby fusing the cMyc epitope tag at its N-terminus (Figure I-36). The epitope tags allow the proteins to be detected by specific antibodies anti-HA and anti-cMyc, respectively (Ferrando *et al*., 2001). Both expression plasmids were introduced into maize protoplasts by electroporation for protein transient expression. As controls, the empty vectors pMENCHU and pMESHI were used. After 15-18 hrs incubation period at 28 $^{\circ}$C in the dark on a shaker (40 rpm), protoplasts were harvested by centrifugation (80 x g, 3 min), proteins were extracted and used for immunoprecipitation assay. For immunoprecipitation and detection of AtGluRS and ABI1, the monoclonal antibodies anti-HA (α-HA), anti-cMyc (α-cMyc) or the polyclonal antibody anti-ABI1-GST (α-ABI1-GST) were used for the immunological assay.
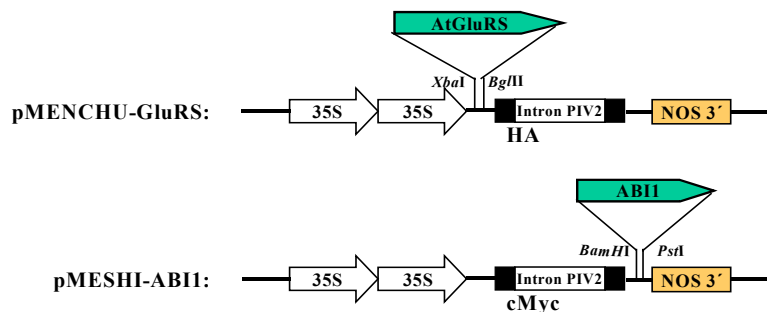
Figure I-36. Schematic diagram of the expression cassettes in pMENCHU-GluRS and pMESHI-ABI1. The *Sma*I-*Bgl*II fragment AtGluRS from pGEX-GluRS was inserted into the filled-in *Xba*I and *Bgl*II sites of pMENCHU. The construct expresses AtGIRS-HA. The *Sma*I-*Pst*I fragment ABI1 from pGAD424-ABI1 was inserted into the filled-in BamHI and PstI sites of pMENSHI. The construct expresses cMyc-ABI1.

Immunological detection of AtGluRS and ABI1

To make sure that the AtGluRS-HA and cMyc-ABI1 were expressed properly and immuno-detected specifically, preliminary experiments were carried out prior to the co-immunoprecipitation experiment. Proteins from the protoplasts transfected with pMENCHU-GluRS or pMESHI-ABI1 were extracted directly with SDS loading buffer and the immuno-detection was performed by western blotting. The anti-HA monoclonal

antibody revealed in extracts of pMENCHU-GluRS transfected protoplasts a single protein band of around 80 kDa corresponding to the molecular weight of AtGluRS-HA (80 kDa), while no signal was detected in the negative control which was transfected with empty vector pMENCHU. The anti-cMyc monoclonal antibody detected in the extracts of protoplasts with expression of cMyc-ABI1 a single band of the size of around 60 kDa (Figure I-37). Interestingly, this size is larger than the expected molecular mass (~49 kDa) of cMyc-ABI1 fusion protein. When the anti-ABI1-GST polyclonal antiserum was used, the 60 kDa-band was also detected only in extracts of protoplasts with expression of cMyc-ABI1. Thus, this band was judged as the specific band corresponding to cMyc-ABI1. In addition, several bands of 70-80 kDa were detected in the cMyc-ABI1 expressed protoplasts as well as in the negative control (Figure I-37). Since the polyclonal antibody was general against the fusion protein ABI1-GST, these unspecific bands might be the GST-related proteins or other unspecifically detectable proteins expressed in any maize protoplasts.
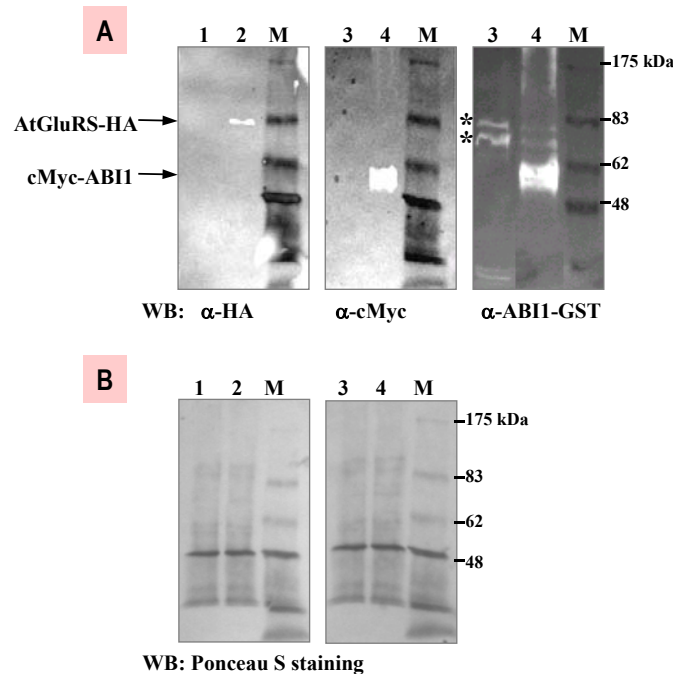
Figure I-37. The transient expression of AtGluRS-HA and cMyc-ABI1 in maize protoplasts. Proteins were extracted from 5 combined transfections. The plasmid DNA used in each single transfection were: (1). 40 µg of pMENCHU; (2). 40 µg of pMENCHU-GluRS; (3). 15 µg of pMESHI; (4). 15 µg of pMESHI-ABI1. Proteins extracted with SDS loading buffer were analyzed by western blots (WB) decorated with anti-HA (α-HA), anti-cMyc (α-cMyc) and anti-ABI1-GST (α-ABI1-GST), respectively. The specific and unspecific (∗) protein bands are indicated (A). Equal loading of the lanes was verified by total protein staining using Ponceau S (B).

Cross detection between the first and second antibodies

Immunoprecipitation was performed by using a primary antibody (*e.g.* the mouse anti-HA) that bind to the antigen of interest (*e.g.* AtGluRS-HA) in a crude protein extracts to form an antibody-antigen complex (Figure I-38). This complex was then incubated with protein A immobilized to the solid support Sepharose. Protein A binds

Figure I-38. Flow chart of co-immunoprecipitation.

Figure I-39. Expected signal on blots of the AtGluRS-HA and cMyc-ABI1 after co-immunoprecipitation detected by different antibody combinations. * indicates the detectable signal.
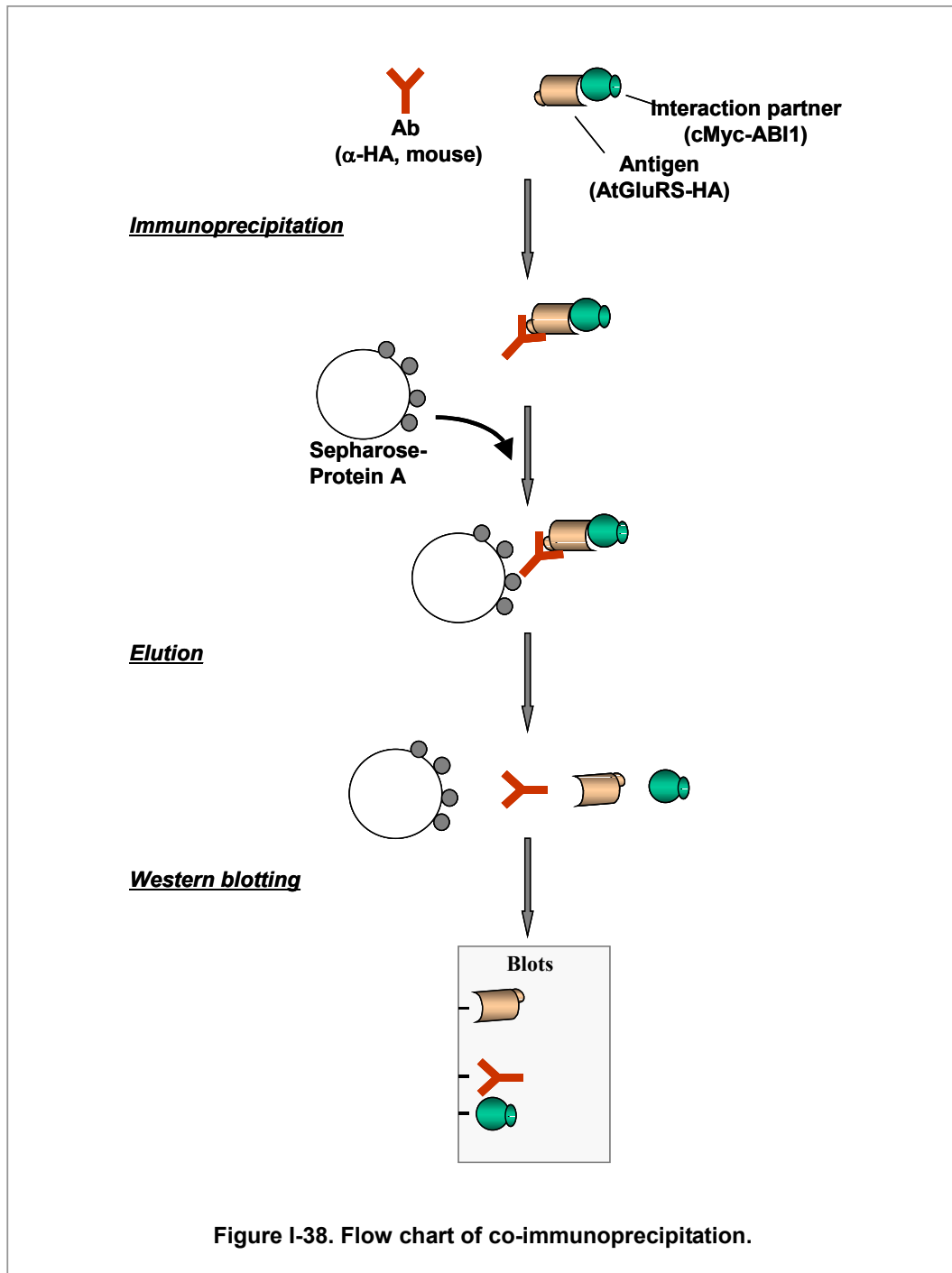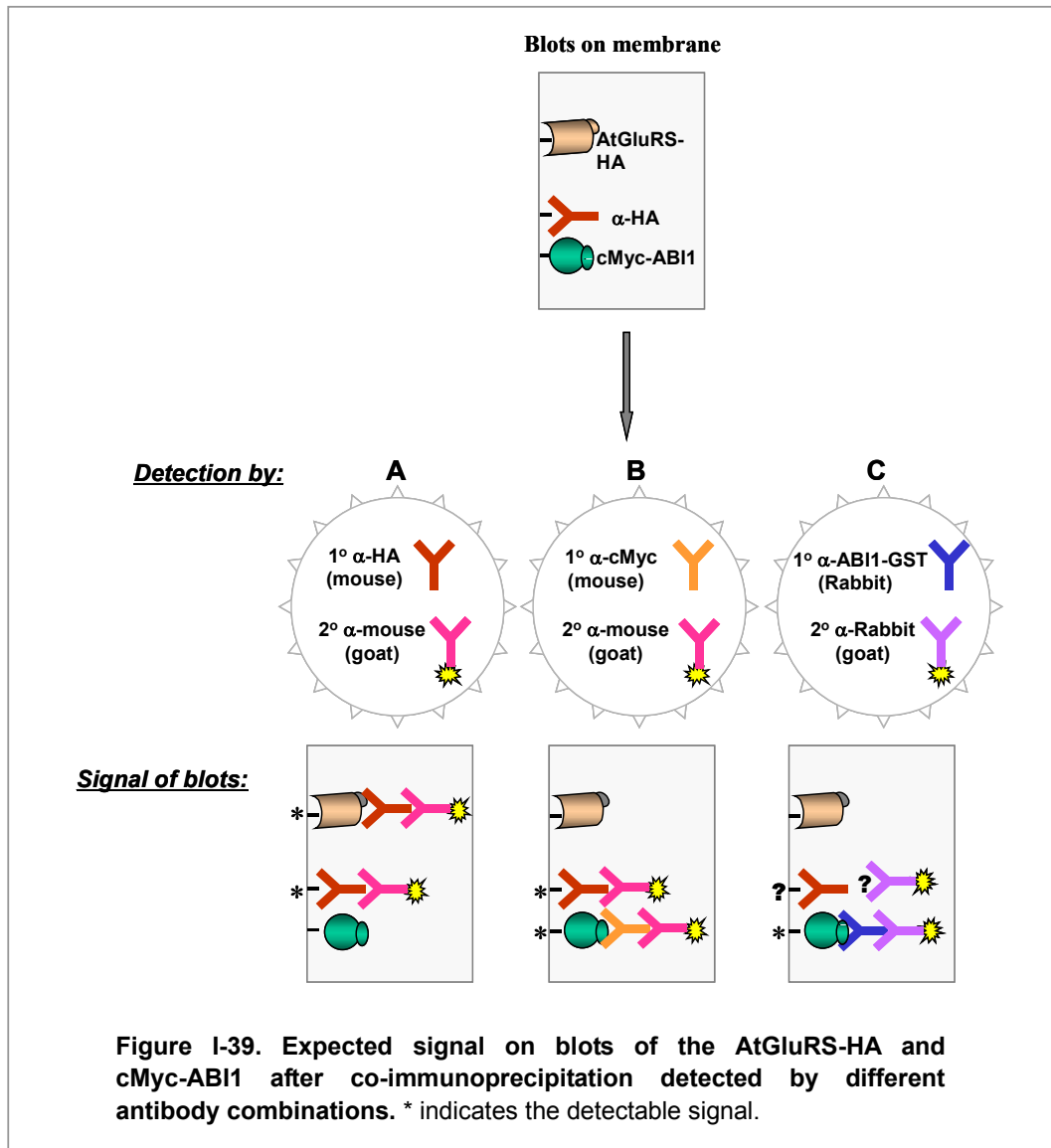
the antibody allowing the entire complex to be separated from the crude mixture by centrifugation. The pellet containing Sepharose-protein A-antibody-protein complex is analysed *via* SDS-PAGE (Figure I-38). The immunoprecipitated protein AtGluRS-HA can then be detected by anti-HA antibody (A in Figure I-39) and the co-immunoprecipitated protein cMyc-ABI1 can be detected by anti-cMyc (B in Figure I-39) or anti-ABI1-GST (C in Figure I-39) combined with the corresponding secondary antibodies, respectively. The primary antibody (*e.g.* mouse anti-HA) in the complex can be detected by the secondary antiserum (*e.g.* goat anti-mouse) which is directly against the primary antibody. Therefore the heavy chain and light chain of the primary antibody will be detected as a protein band of 50 kDa and 25 kDa, respectively (A and B in

Figure I-39), and with interference with the detection of proteins of similar molecular mass, *e.g.* the cMyc-ABI1 of 60 kDa in this experiment (B in Figure I-39). To avoid this problem, an primary antibody from a different source (rabbit) was used for detection. The secondary antibody directly against this antibody (goat anti-rabbit) was used together (C in Figure I-39). To analyse the cross-interactions of primary antibody anti-HA with different secondary antibodies, the anti-HA antibody was blotted on a nitrocellulose membrane after SDS-PAGE and detected by goat anti-mouse or goat anti-rabbit antibodies. Figure I-40 shows that 4 to 100 ng of the anti-HA monoclonal antibody from mouse were not detected by the antibody goat anti-rabbit conjugated with horseradish peroxidase (HRP). In contrast, the anti-HA monoclonal antibody from mouse was clearly detected by the goat anti-mouse-HRP antibody. Thus the combination of secondary goat anti-rabbit antibody and primary rabbit polyclonal antibody could be used for detection of the proteins co-immunoprecipitated by mouse anti-HA without significant interference.

Expression levels of AtGluRS-HA and cMyc-ABI1

Comparable expression of the two partner proteins is crucial for obtaining good co-immunoprecipitation results. Thus, it is advisable to quantify the AtGluRS-HA and cMyc-ABI1 protein prior to the co-immunoprecipitation experiment.

The signal of AtGluRS-HA protein from immunological detection is achieved *via* detecting the HA-epitope-bound primary α-HA by the secondary antibody goat anti-mouse-HRP. Thus, the amount of bound α-HA is determined. Hence, the protein anti-HA antibody can be used as the standard to quantify the precipitated protein AtGluRS-HA. Similarly, the protein anti-cMyc antibody can be also used for quantification of the cMyc-ABI1.

To quantify the expression level of AtGluRS-HA and cMyc-ABI1, extracts from 3 transfections for expression of each protein were assayed. In each transfection, 40 µg of pMENCHU-GluRS, 5 µg or 15 µg of pMESHI-ABI1 were used, respectively. The results showed that the intensity of the AtGluRS-HA band was comparable to that of 0.1-0.5 ng anti-HA antibody bands. In addition the signal of cMyc-ABI1 from 5 µg DNA and from 15 µg DNA was comparable to that of 0.5-1 ng and over 10 ng anti-cMyc antibody, respectively (Figure I-41). Thus, transfection of 40 µg of pMENCHU-GluRS yielded the same amount of recombinant protein as that of 5 µg of pMESHI-ABI1.

Amount of anti-HA monoclonal antibody (ng): 100 20 4   100 20 4

83 kDa
62
48

WB detected by : anti-Mouse-HRP   anti-Rabbit-HRP
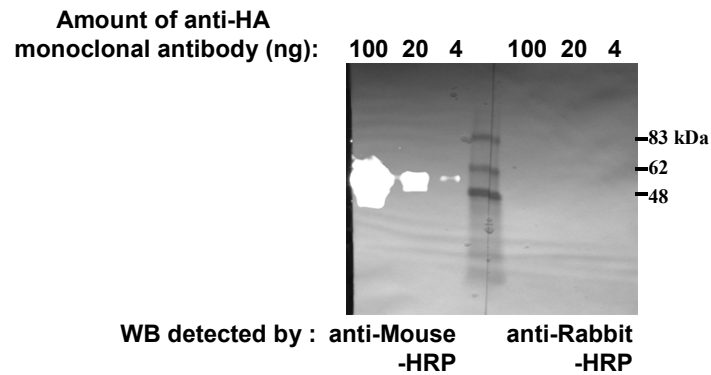
Figure I-40. Cross-detection of monoclonal mouse anti-HA and different secondary antibodies. 4, 20 and 100 ng of anti-HA antibody were blotted onto a nitrocellulose membrane after SDS-PAGE and detected by goat anti-mouse and goat anti-rabbit antibodies, respectively, using light emission catalyzed by horseradish peroxidase (HRP).

α-HA (ng)

A   0.01 0.1 0.5 1 10

AtGluRS-HA →

← α-HA

WB: α-HA, anti-mouse-HRP

α-cMyc (ng)

B   C   0.01 0.1 0.5 1 10

cMyc-ABI1 →

← α-cMyc

WB: α-cMyc, anti-mouse-HRP
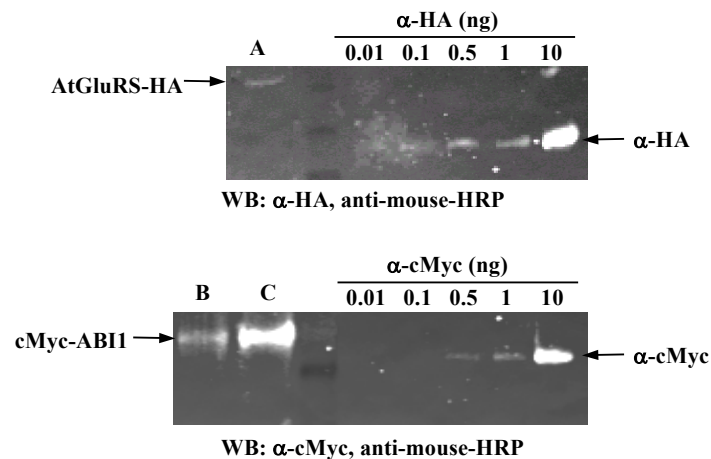
Figure I-41. Quantification of AtGluRS-HA and cMyc-ABI1. Different amount of anti-HA or anti-cMyc antibodies, and protein extracts from transfected protoplasts were subjected to SDS-PAGE. The western blots were performed with the corresponding primary antibodies and the goat anti-mouse-HRP. Proteins were extracted from three homolgous transfections each with 40 µg of pMENCHU-GluRS (A), 5 µg of pMESHI-ABI1 (B) or 15 µg of pMESHI-ABI1 (C).

Protection of AtGluRS from degradation by PMSF

PMSF (phenyl methyl sulforyl fluoride) protects proteins from degradation by serine proteases. My results showed that the AtGluRS-HA was detected in the immunoprecipitated fraction after 2hrs-immunoprecipitation when 1 mM of PMSF was included in the incubation buffer, but could not be detected when PMSF was omitted (Figure I-42A). Furthermore, even though the general protease inhibitor "cocktail" was included in the extraction buffer, the lack of PMSF resulted in a detectable protein signal using α-HA from extracts of AtGluRS-HA expressing protoplasts in molecular mass of around 48 kDa rather than 80 kDa (Figure I-42B). Those smaller molecular mass proteins should be the degradation products of AtGluRS. The results indicate that PMSF can efficiently protect the AtGluRS from protein degradation.
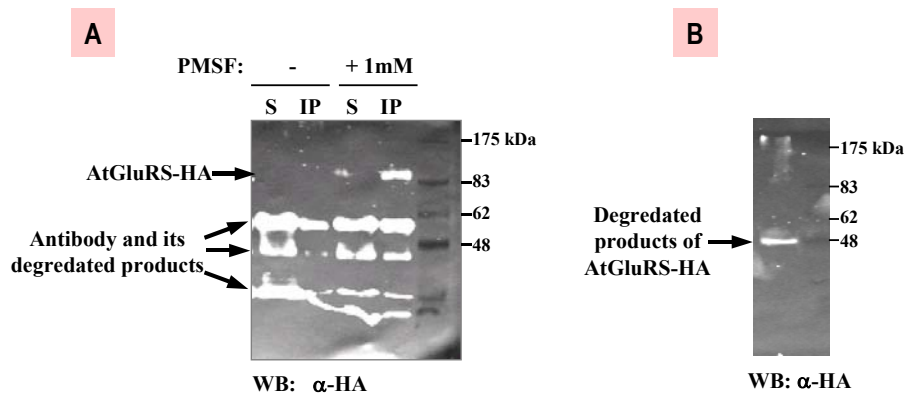
Figure I-42. PMSF protects the AtGluRS-HA from degradation. (A). Protein immunoprecipitation in the absence or presence of PMSF. Protein extracts from the AtGluRS-expressed protoplasts was subjected to immunoprecipitation by incubating with anti-HA antibody for 1 hour and with sephorase-protein A for another hour on ice. The supernatant (S) and the immunoprecipted fraction (IP) after immunoprecipitation was analyzed by western blots using anti-HA antibody. (B). Protein extracted from AtGluRS-expressing protoplasts by a extraction buffer without additional PMSF showed that the detectable HA-fusion protein had a molecular mass of around 48 kDa which is smaller than the predicted Mr of approx. 80 kDa.

Co-immunoprecipitation of AtGluRS-HA and cMyc-ABI1

Pilot control experiments showed that the cMyc-ABI1 was not precipitated by the Sepharose-protein A (Figure I-43), revealing no background adsorption of the supposed second co-immunoprecipitated partner cMyc-ABI1 on the solid support. To perform the co-immunoprecipitation, the AtGluRS-HA was used as the immunoprecipitation target. Crude protein extracts (designated as **C** fraction) from the transfected maize protoplasts

were incubated with anti-HA antibody for 1 hour and subsequently, with Sepharose-protein A for another hour. After a centrifugation step the supernatant was collected (fraction **S**). The pellet was washed once with IP buffer and the supernatant was collected (**W** fraction). The remaining pellet (Sepharose - protein A - anti-HA antibody - HA-tagged protein and its associated partners) was eluted by SDS loading buffer (**IP** fraction) (Figure I-9). Western blots of all those obtained fractions using comparable aliquots of initial extracts were performed by anti-HA antibody or anti-ABI1-GST antibody respectively, each antibody combined with their corresponding secondary antibody. The results (Figure I-44A) showed that the majority of expressed AtGluRS-HA from extracts of protoplasts with expression of AtGluRS and ABI1 or AtGluRS alone was present in the IP fraction. No AtGluRS-HA band was detected in the extracts from the protoplasts with cMyc-ABI1 only. Evaluation of the signals by using the *SimplePCI* program revealed that 84% and 68% of the AtGluRS-HA could be recovered from protoplasts expressing AtGluRS-HA / cMyc-ABI1 or AtGluRS alone. It highlights the high specificity and efficiency of the immunoprecipitation of AtGluRS-HA by anti-HA antibody. When using anti-ABI1-GST to detect the ABI1 protein from protoplasts with co-expression of AtGluRS and ABI1, 64% of expressed cMyc-ABI1 was detected in the S fraction, and 23% of it was recovered in the IP fraction together with AtGluRS-HA.

In the extracts of the protoplasts with expression of cMyc-ABI1 alone, majority of the cMyc-ABI1 protein was recovered in the S fraction (> 90%), and the rest 10% was undetectable might present in the wash fraction. It indicates that the cMyc-ABI1 was not precipitated in the absence of AtGluRS-HA (Figure I-44). Reproducible results were obtained in an independent experiment and showed that over 25% of ABI1 from extracts of protoplasts expressing AtGluRS-HA / cMyc-ABI1 was found in the IP fraction (Figure I-45).

WB: α-ABI1-GST
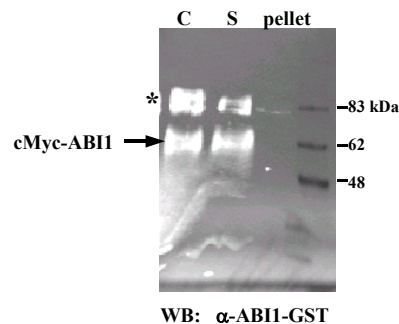
Figure I-43. No adsorption of cMyc-ABI1 on the sepharose-protein A. Protein extracts from protoplasts with expression of cMyc-ABI1 were incubated with sepharose protein A for 2 hours on ice and then the supernatant (S), pellet, as well as the same amount of crude extract (C) was detected by western blot using anti-ABI1-GST. ∗ indicates unspecific band.
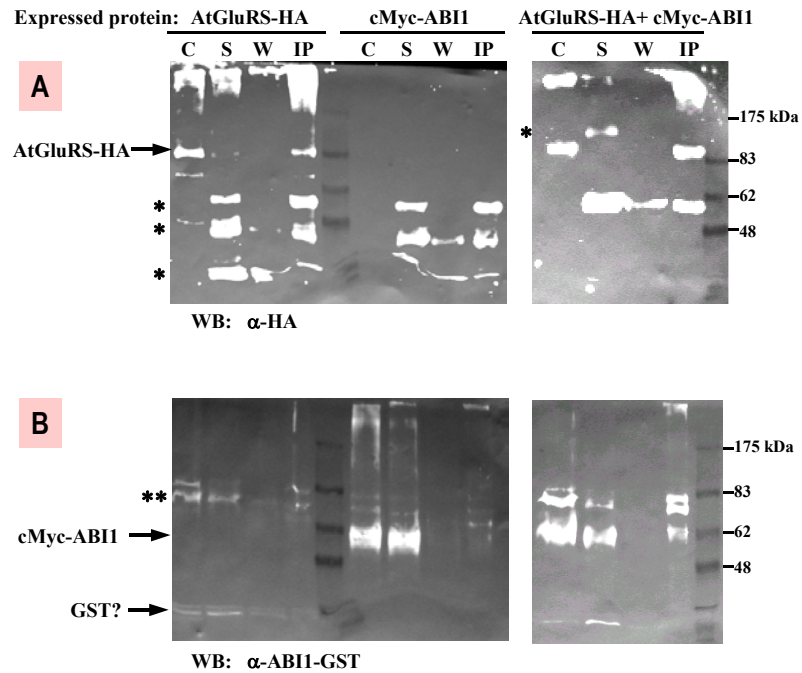
Figure I-44. Protein interaction between AtGluRS-HA and cMyc-ABI1 in maize protoplast demonstrated by co-immunoprecipitation. Protein extracts were prepared from protoplasts expressing AtGluRS-HA and cMyc-ABI1 individually or both together. The immunoprecipitation reaction was performed by using anti-HA antibody. Fractions of the crude extract (C), supernatant (S), wash fraction (W) and the immunoprecipitated fraction (IP) were analyzed by western blotting. Blots in (A) were decorated with anti-HA and goat anti-mouse-HRP, and blots in (B) were detected by anti-ABI1-GST and goat anti-rabbit-HRP. ∗ indicates a contaminating band generated by the antibody and the solid support. ∗∗ indicates another unspecific band.
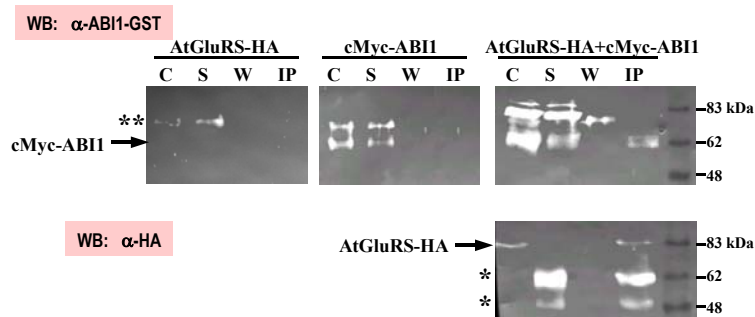
Figure I-45. Interaction of AtGluRS-HA and cMyc-ABI1 in maize protoplasts demonstrated by Co-immunoprecipitation. Details was found in Figure I-44.

These data show that the cMyc-ABI1 is co-immunoprecipitated with AtGluRS-HA. It demonstrates that the interactions between AtGluRS and ABI1 in yeast and *in vitro* reflect the ability of both proteins to associate in plant cells as well.

3.2.5 Ternary interaction among AtHB6, AtGluRS and a third protein in three-hybrid yeast system

The results obtained from the yeast two-hybrid system and the *in vitro* and *in vivo* interaction assay strongly suggested that interactions occur between AtHB6 and AtGluRS, ABI1 and AtHB6, and ABI1 (or ABI2) and AtGluRS. These protein cross-interactions seem to be complex and could indicate the formation of a ternary protein complex. In yeast, GluRS was reported to be part of a multisynthetase complex with Arc1p and the methionyl-tRNA synthetase (MetRS) (Galani *et al*., 2001). However, less is known about plant *Arabidopsis* AtGluRS with respect to its putative interactions. *Arabidopsis* AtGluRS seems to form higher complexes with other proteins, or interacts with other proteins competitively. In the following experiment I tried to investigate this question by using a three-hybrid interaction analysis.

Establishment of three-hybrid system

Constructions:

The basic vectors used in this three-hybrid system are pACT2 and pBridge. GluRS(1-261) was fused to AD in the pACT2 vector. And two different cDNA fragments (*e.g*., X, Y) were cloned into pBridge to generate constructs pB/X//Y and allow expression of a DBD-fusion protein (X) as well as a third protein (Y), the expression of the later is controlled by P_{met25} thus can be regulated by the methionine level. In this experiment, AtHB6dC269 was cloned into the multiple cloning site MCS I of pBridge as the X to produce the DBD-fusion protein DBD-AtHB6dC269, and ABI1, abi1, ABI2, abi2, AtHB6dC269 or AtHB7dc136 was cloned into the MCS II of pBridge as the Y to provide repressible expression of a third protein (Figure I-45). The details of cloning were as described in Materials and Methods. The effects of the third protein on the interaction between DBD-AtHB6dC269 and AD-AtGluRS(1-261) were then evaluated by measuring the β-galactosidase activity and cells growth in selective medium.

Expression level of the third protein controlled by methionine:

To demonstrate how the expression of the third protein was controlled, the yeast cells transformed with pB/HB6dC269//ABI1 were cultivated in selective SD medium having different methionine concentrations, and the protein assay was performed.
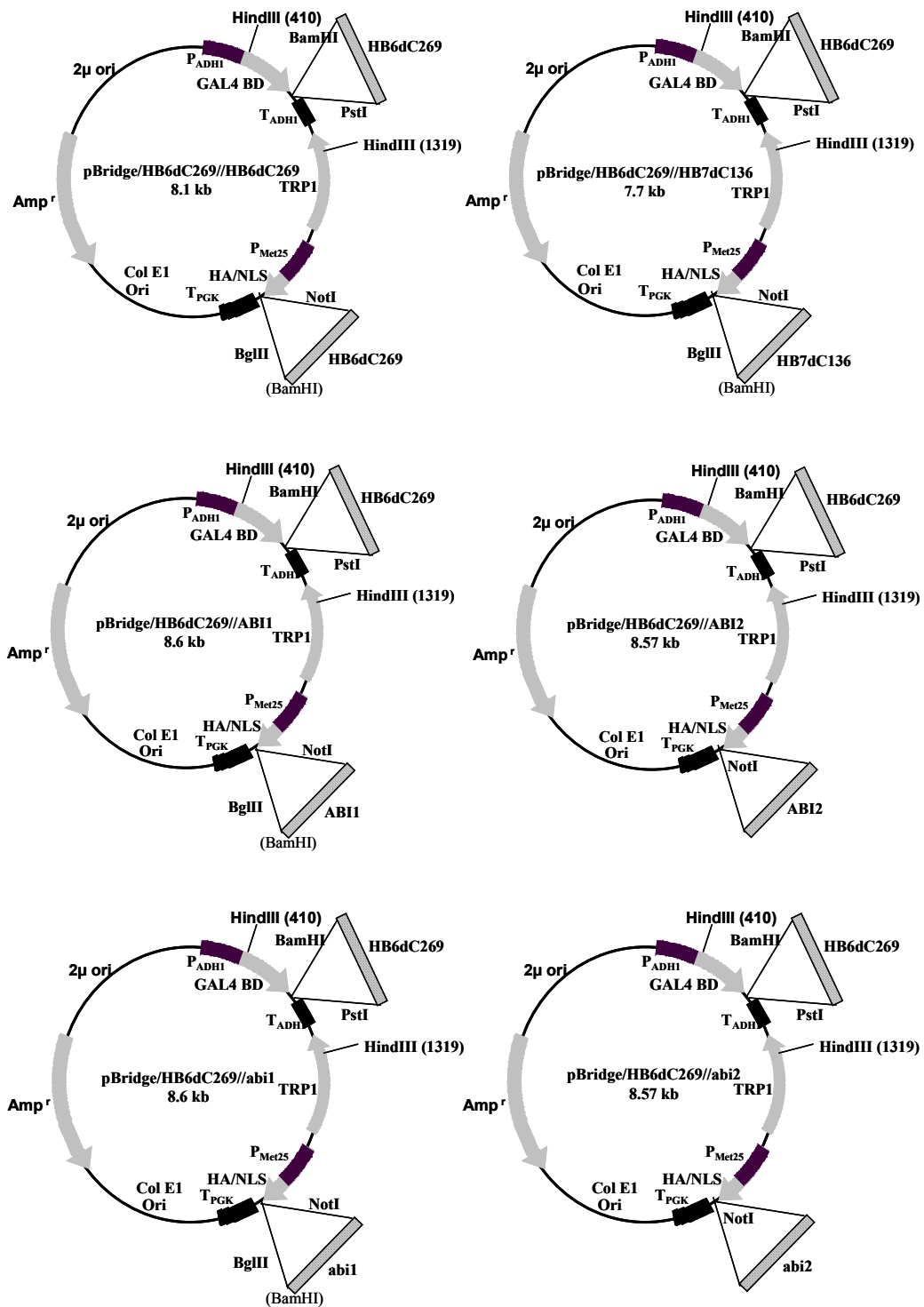
Figure I-45. Constructs for three-hybrid system.

Three methionine concentrations were used in the experiment to regulate the conditional methionine promoter (P_{Met25}) and control the third protein's expression (ABI1): without methionine, where the expression is switched on; 1 mM methionine, where the expression is expected to be completely inhibited; and 0.15 mM methionine, which is the optimal methionine concentration for normal yeast cultivation, and was used to obtain partial expression.

The [pBrige/ABI1dN, pACT2-ABI1fl]-co-tansformed cells were used to check the expression level of DBD-fusion and AD-fusion proteins at 0.15 mM and 1 mM methionine. Total protein was extracted by the TCA method, and the proteins were seperated by SDS-polyacrylamide gel electrophoresis and then subjected to western blot analysis. The transferred proteins were stained using Ponceau S to show total proteins. The AD-DBD-ABI1 fusion protein, and (P_{Met25})-ABI1 was detected using the anti-ABI1-GST antibodies (Figure I-46). The expression of the third protein (P_{Met25})-ABI1 could be repressed to some extent when 0.15 mM or 1 mM methionine were supplied in the medium; however, this repression was not complete, since the signal was still detectable with the residue of approximately 25% and 10%, respectively. There were no differences between the expression of AD-ABI1 or DBD-ABI1dN at different methionine levels (Figure I-46), indicating the expression of AD- and DBD-fusion was not affected by the methionine level.
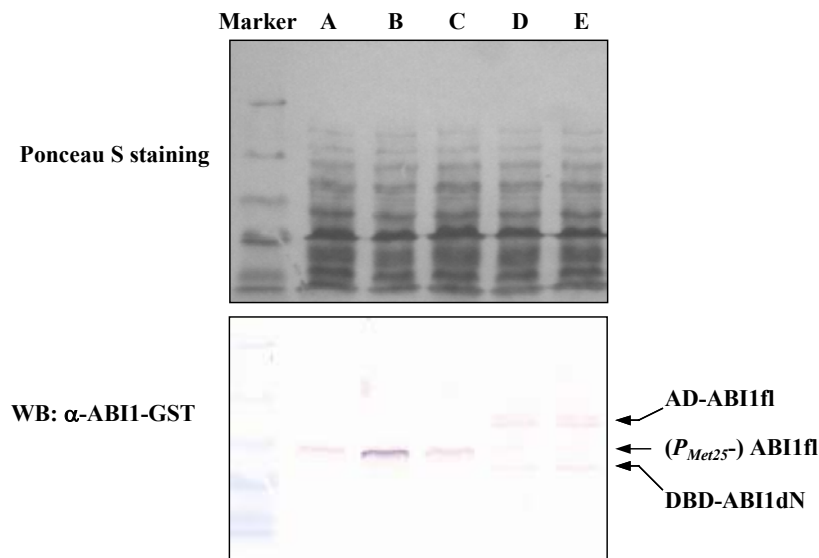
Figure I-46. The expression level of ABI1 protein in yeast three-hybrid system. A, B, C corresponds to the protein extracted from pB/HB6dC269//ABI1fl-transformed yeast cells which were cultivated in the SD medium with 1 mM, 0 mM and 0.15 mM methionine, respectively. D and E corresponds to the protein extracted from [pB/ABI1dN, pACT2-ABI1fl]-transformed yeast cells grown in SD medium with 0.15 mM and 1 mM methionine, respectively.

AtHB6dC269 competes with AtHB6dC269 in interacting with AtGluRS(1-261):

HF7c yeast cells co-transformed with pB/HB6dC269//HB6dC269 (or the control, pB/HB6dC269) and pACT2-GluRS(1-261) were cultivated in the absence or in the presence of 0.15 mM or 1 mM methionine. In this experiment, the P_{Met25} controlled protein AtHB6dC269 (functioning as the third protein) was expected to compete with DBD-AtHB6dC269 in interactions with AD-AtGluRS(1-261). A minimal cultivation medium lacking leucine and tryptophan was used to select for both plasmids and, lacking methionine, to permit expression by the P_{Met25} promoter.
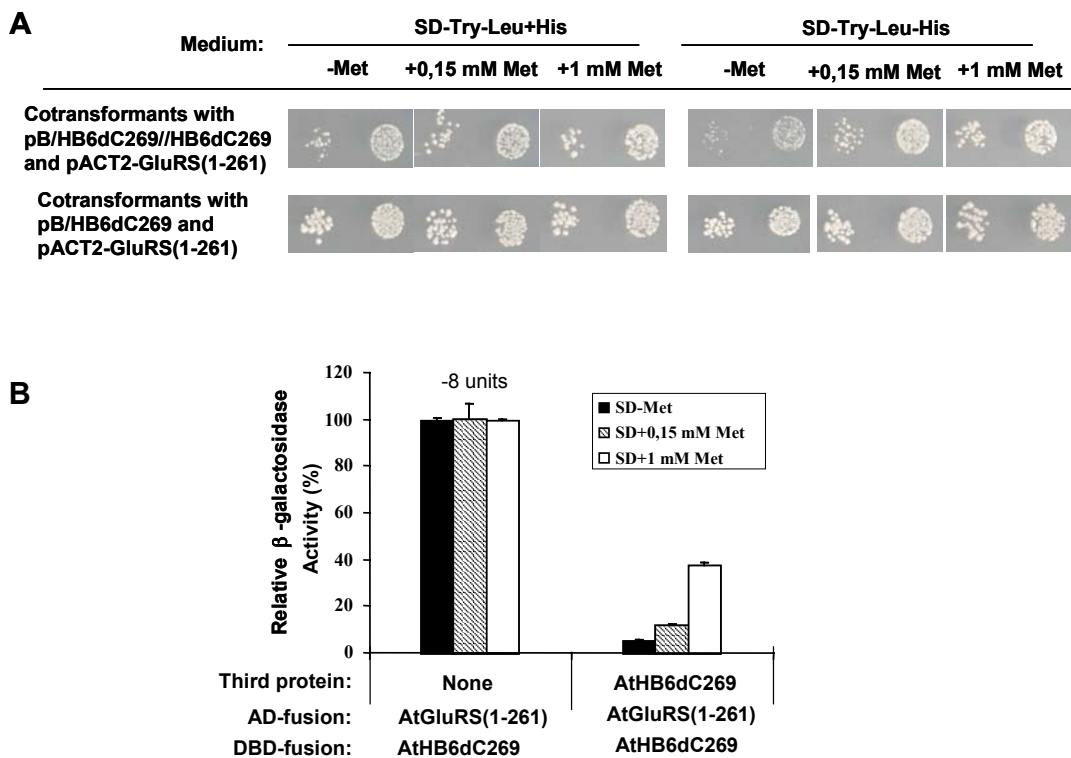
Figure I-47. The effect of the third protein AtHB6dC269 on the interaction between DBD-AtHB6dC269 and AD-AtGluRS(1-261). A: Yeast HF7c cells co-transformed by pACT2-GluRS(1-261) and pB/HB6dC269//HB6dC269 (or pB/HB6dC269, as the control) were grown on minimal SD selective medium spotted at 1X and 10X dilutions. Yeast growth was performed over 3 days at 30 °C. B: β-galactosidase assay. Yeast cells co-transformed with pACT2-GluRS(1-261) and pB/HB6dC269//HB6dC269 (or as the control, pB/HB6dC269) were grown in a minimal selective medium containing different concentrations of methionine to mid exponential phase, then the cells were collected for determination of β-galactosidase activity.

[pB/HB6dC269//HB6dC269, pACT2-GluRS(1-261)]- transformed cells on the medium without methionine exhibited weaker growth in the absence of histidine than in the presence of histidine (Figure I-47A), indicating that the interaction of AD-AtHB6dC261 and DBD-AtHB6dC269 was repressed by the third protein AtHB6dC269 therefore it could not activate the *His3* reporter gene efficiently. However, the controlling expression of the third protein AtHB6dC269 by the application of 0.15 mM or 1 mM methionine rescued this inhibition of growth. In the control cells transformed with pB/HB6dC269 and pACT2-GluRS(1-261), the methionine level did not affect cell growth.

To further demonstrate the influence of a third protein on the reporter gene's transcriptional activation, a β-galactosidase assay was performed (Figure I-47B). The enzyme activity of the [pB/HB6dC269, pACT2-GluRS(1-261)]- transformed cells (as the control) was approximately 8 units at any methionine concentration examined. In contrast, the enzyme activity of [pB/HB6dC269//HB6dC269, pACT2-GluRS(1-261)]- transformed cells was apparently affected by methionine. In the absence of methionine, a more than 90% reduction of β-galactosidase activity was obtained when compared with the control transformants. In cells grown with methionine, the enzyme activity was partially rescued. Compared with transformants grown in the absence of methionine, the cells grown in the presence of 0.15 mM methionine exhibited a two-fold increase in enzyme activity, and transformants grown in the presence of 1 mM methionine had an approximately 5-fold increase in enzyme activity. However, high levels of methionine (1 mM) was not able to completely recover the transcriptional activation of the reporter gene and only resulted in approximately 38% of the enzyme activity seen in the [pB/HB6dC269, pACT2-GluRS(1-261)]- transformants.

Suppression of the interaction between AtHB6dC269 and GluRS(1-261) by AtHB7

When AtHB7dC136 was expressed as the third protein in pBridge, regardless of the absence or presence of histidine or the methionine in the medium, the [pB/HB6dC269//HB7dC136, pACT2-GluRS(1-261)]- transformed cells could grow as well as the [pB/HB6dC269, pACT2-GluRS(1-261)]- transformed cells did (Figure I-48A). It seems that the expression of AtHB7dC136 did not severely interfere with the interaction between DBD-AtHB6dC269 and AD-AtGluRS(1-261) in terms of the expression of the *His3* reporter gene.

The β-galactosidase assay demonstrated that (Figure I-48B), when grown in the medium containing 0.15 mM methionine or without methionine, the [pB/HB6dC269//HB7dC136, pACT2-GluRS(1-261)]- transformed cells had

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approximately half the enzyme activity as the control cells not expressing the third protein. When the expression of AtHB7dC136 was repressed in the presence of 1 mM methionine, the [pB/HB6dC269//HB7dC136, pACT2-GluRS(1-261)]- transformed cells had approximately 80% of the enzyme activity of the control [pB/HB6dC269, pACT2-GluRS(1-261)]- transformed cells.
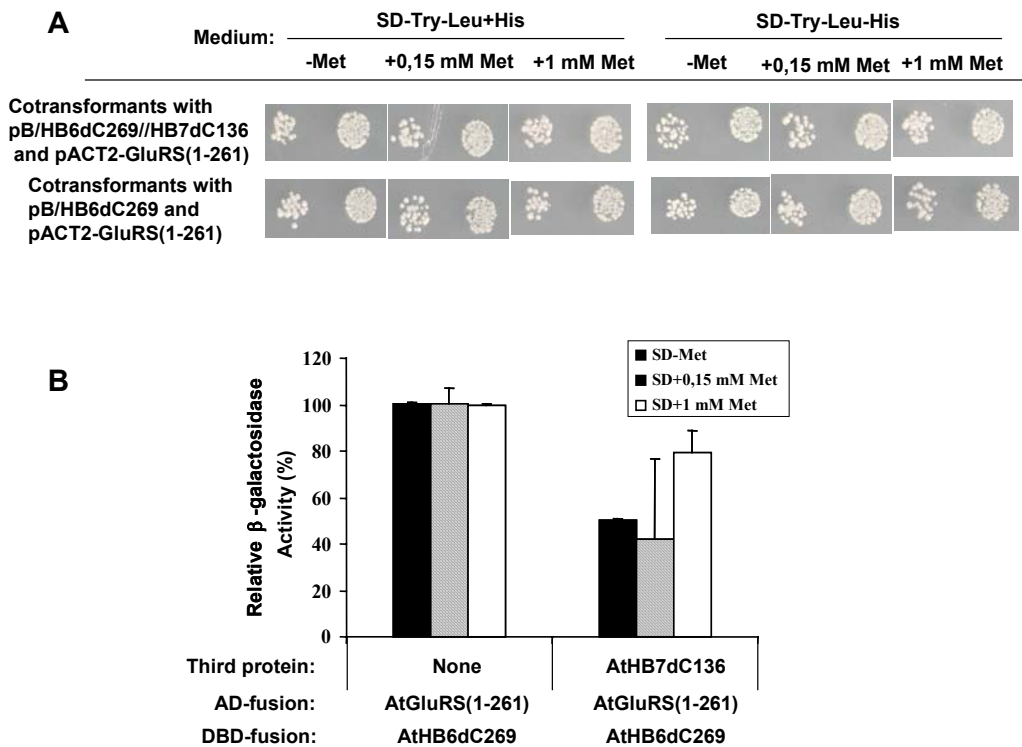
Figure I-48. The effect of the third protein AtHB7dC136 on the interaction between DBD-AtHB6dC269 and AD-AtGluRS(1-261). The cell growth assay (A) and β-galactosidase assay (B) of the co-transformants [pACT2-GluRS(1-261), pB/HB6dC269//HB7dC136 (or pB/HB6dC269, as the control)] were performed as illustrated in Figure I-47.

Suppression of the interaction between AtHB6dC269 and GluRS(1-261) by ABI1/ ABI2

When ABI1 and ABI2 were introduced under the control of the P_{Met25} promoter, the co-tansformants with pB/HB6dC269//ABI1 (or ABI2) and pACT2-GluRS(1-261) did not grow in the absence of methionine and grew only weakly in the presence of 0.15 mM methionine when the medium contained no histidine. However, application of 1 mM methionine allowed these transformants to grow well. When abi1 and abi2 were used as third proteins, active growth was observed in the cells grown on the media with

or without histidine at any methionine concentration examined (Figure I-49). These results indicate that ABI1 and ABI2 interfered with the interaction between DBD-AtHB6dC269 and AD-AtGluRS(1-261) and affected the activation of the *His3* reporter gene transcription, however, this was not the case for abi1 and abi2.

The co-transformants [pB/HB6dC269//ABI1 (or ABI2) and pACT2-GluRS(1-261)] grew weakly on the medium without methionine even when histidine was applied. A possible reason might be that the protein phosphatase 2C activities of ABI1 or ABI2 somehow affects yeast growth, subsequently the cells were less receptive to methionine application to remedy the negative effect.

To quantify the effect of the third-protein ABI1, ABI2, abi1 or abi2 on the interaction between DBD-AtHB6dC269 and AD-AtGluRS(1-261), a β-galactosidase assay was performed (Figure I-50). When ABI1 was introduced as the third protein, only background level of the β-galactosidase activity was detected. Suppression of ABI1 expression by the application of 0.15 mM or 1 mM of methionine rescued the reporter activity to approximately 10% and 40% of the controls, respectively. When ABI2 was introduced, β-galactosidase activity of the transformants was reduced by more than 90%. The negative interference of ABI2 could not significantly be relieved by methionine application. These results indicate that ABI2, as the third protein, severely disturbed the interaction between AtHB6dC269 and AtGluRS(1-261).

Compared with wild type ABI1 or ABI2, the mutant type abi1 or abi2 as the third-protein had less influence on the interaction between DBD-AtHB6dC269 and AD-GluRS(1-261), β-galactosidase activity being only reduced to about 40% and 15% in the absence of methionine, respectively. When methionine (0.15 mM, 1 mM) was applied to suppress the expression of abi1 or abi2, approximately 75% and 40% of the control enzyme activity were obtained respectively in the [pB/HB6dC269//*abi1*, pACT2-GluRS(1-261)]-transformed and [pB/HB6dC269//*abi2*, pACT2-GluRS(1-261)] -transformed cells.

In view of the above results, ABI1 and ABI2 apparently interfered with the interaction between AtHB6dC269 and AtGluRS(1-261), while abi1 and abi2 had relatively weaker, but still notable effects. Bearing in mind the observation of that AtHB6dC269 interacts with ABI1, abi1, ABI2 and abi2 very weakly, it seems that the AtHB6 and PP2C are most probably competing in the interaction with AtGluRS. However, it is still hard to eliminate a possible role of protein dephosphorylation in this scenario if we take into consideration of that the AtGluRS(1-261) made no discrimination in interaction with ABI1 and abi1, while the ABI1 give stronger repression on the interaction of AtGluRS(1-261) and AtHB6dC269 than the abi1.

Medium	SD-Try-Leu+His						SD-Try-Leu-His					
	-Met		+0,15 mM Met		+1 mM Met		-Met		+0,15 mM Met		+1 mM Met	
Third-protein: ABI1												
abi1												
ABI2												
abi2												
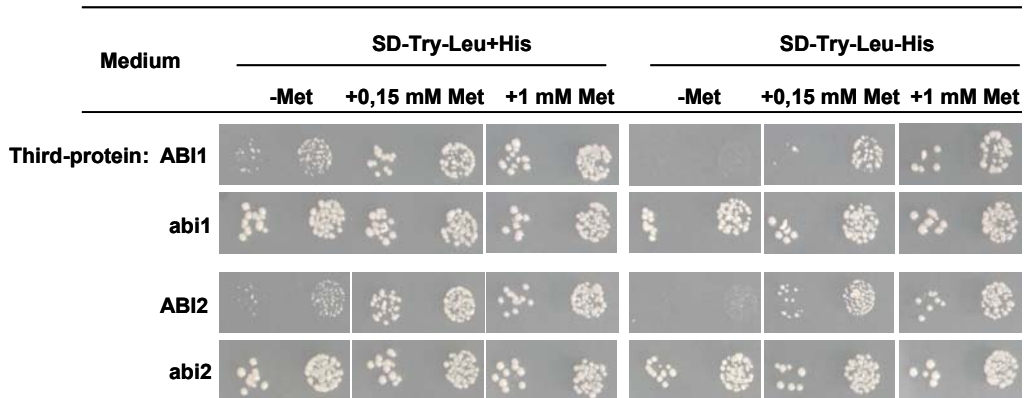
Figure I-49. Cells growth of the yeast transformants [pACT2-GluRS(1-261), pB/HB6dC269//ABI1(or abi1, ABI2, abi2)]. Transformants were cultivated on minimal SD selective medium spotted at 1x and 10x dilutions. Yeast growth was performed over 3 days at 30 ^{o}C in the absence or presence of 0.15 mM and 1 mM methionine, controlling the expression of the third partner.
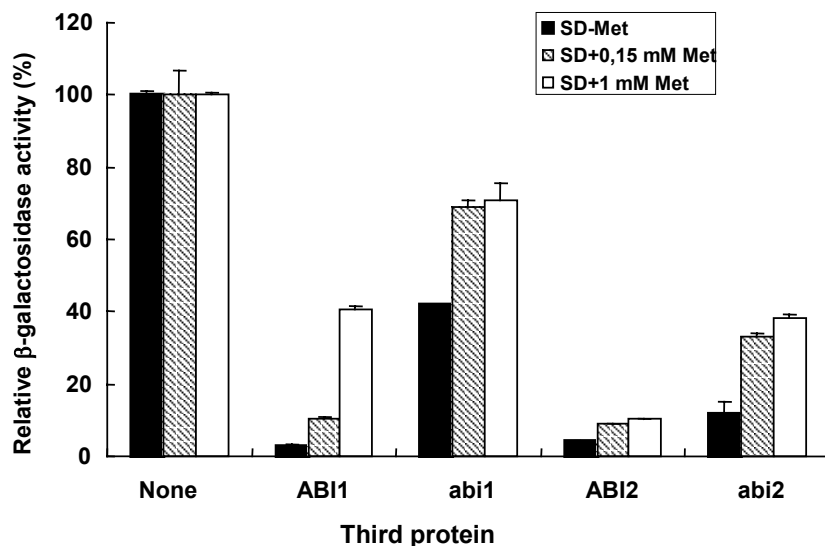
Figure I-50. Quantitation of the effect of a third protein ABI1, abi1, ABI2 or abi2 on the interaction of AtHB6dC269 and AtGluRS(1-261). β-Galactosidase assay. Co-transformants were grown in a minimal selective medium containing different concentrations of methionine to mid exponential phase, and then the cells were collected for determining of β-galactosidase activity. The data is presented as the average of 3 independent assay. Comparable results were yielded in two independent experiments.

In the yeast two-hybrid system, interaction between full-length AtGluRS and AtHB6, AtHB7, or ABI1 could not be detected. Considering that a third protein might alter the property of AtGluRS for interaction in some way, *e.g.* through modification (Tirode *et al.*, 1997), either AtHB7dC136 or ABI1 was expressed as the third-interaction partner in the yeast three-hybrid. Neither ABI1 nor AtHB7 enhanced the interaction of AtHB6 and AtGluRS as judged from the expression of reporter gene (Figure I-51).
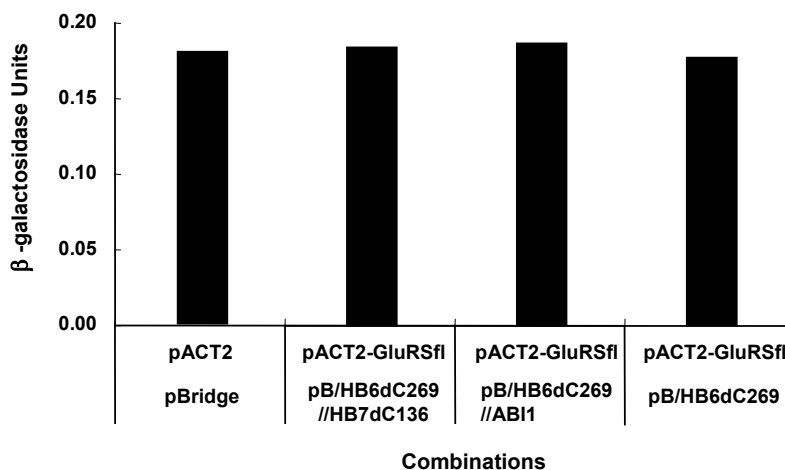
Figure I-51. Quantitation assay of the interaction of AtGluRSfl and AtHB6dC269 in the presence of a third protein ABI1 or AtHB7dC136. Yeast cells co-transformed with the different combinations of plasmids as indicated were grown in a minimal selective medium without methionine to mid exponential phase, and then the determination of the β-galactosidase activity was performed. All the enzyme activity of the combinations were as low as the negative control [pACT2, pBridge].

3.3 Identification of interaction partners of AtGluRS by a yeast two-hybrid system

Since the present results strongly suggested multi-protein interactions among AtGluRS, AtHB6 and ABI1, an additional yeast two-hybrid screen with AtGluRS was carried out to search for further components of protein interaction.

3.3.1 Different versions of bait proteins

Two AtGluRS versions were cloned in pGBT9: the full-length AtGluRS and the N-terminal of AtGluRS(1-261) (Figure I-52).

Before being used as baits to screen the library, the pGBT9-GluRS(1-261) and pGBT9-GluRSfl were transformed to yeast HF7c with pGAD424 to test the expression of the *His3* and *LacZ* reporter genes. The results demonstrated that these transformants could not grow on the medium without histidine. The liquid assay for β-galactosidase

also revealed that they had only very low enzyme activity that was comparable to the background (Figure I-53). These results indicated that GluRS(1-261) and GluRSfl could not activate the transcription of the reporter genes autonomously, and they were suitable to be used as baits to screen the library.
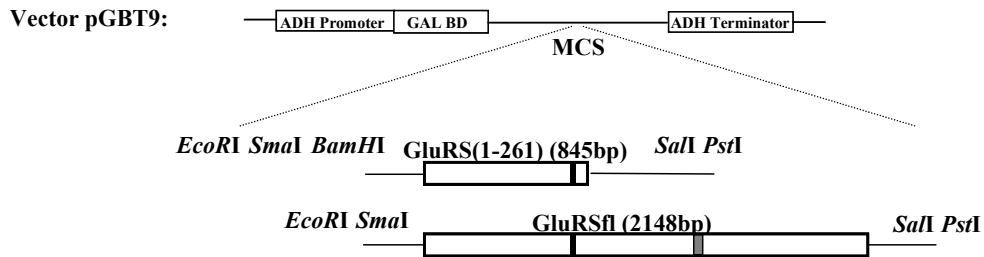
Figure I-52. Schematic view of pGBT9-GluRS(1-261) and pGBT9-GluRSfl constructs used for screening. The dark boxes represent the putative ATP binding sites "HIGH" motifs, the grey box represents the motif 'KMSKS' which is the putative binding site of the 3' end of the tRNA.
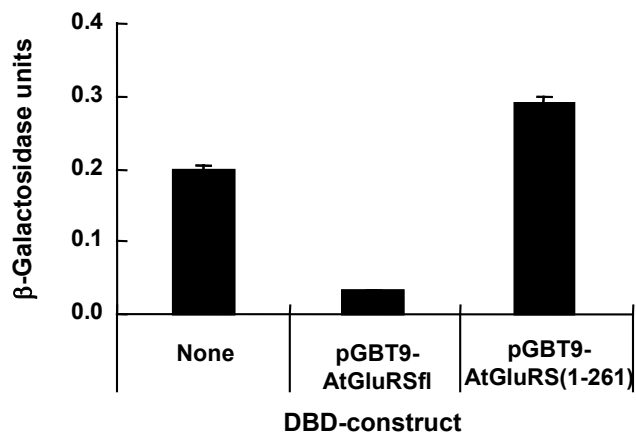
Figure I-53. None auto-activation of pGBT9-GluRS(1-261) or pGBT9-GluRSfl in yeast two-hybrid system. Yeast cells co-tansformed with empty AD-plasmid pGAD424 and pGBT9-GluRS(1-261) or pGBT9-GluRSfl were assayed for β-galactosidase activity.

3.3.2 Interaction partners of AtGluRS

The [pGBT9-GluRS(1-261)]-transformed yeast strain HF7c was subsequently transformed with the Ohio *Arabidopsis* cDNA library. In this transformation, 100 μg

library DNA produced $6,4\times10^6$ transformants. From this transformant pool, 49 *His3* positives were obtained and 5 of them were *LacZ* positive. Sequence analysis of these candidates (see Appendix 4.) and similarity search using BLAST (Altschul *et al.*, 1997) revealed that:

1. Candidate S1: identical to the *Arabidopsis thaliana* rRNA repeat unit (emb: X52322.1). The predicted fusion protein encoded from the start code of AD has a high consensus to a senescence-associated protein ssa-13 in *Pisum sativum*. (Pariasca *et al.*, 2001).

2. Candidate S47: identical to the mRNA of At3g41970 gene which was postulated to be a product of an unprocessed transcript (gb accession number AY090960; Yamada *et al.*, unpublished). It was inserted in a false-orientation. The predicted fusion protein encoded from the start code of AD also has a high consensus to a senescence-associated protein ssa-13 in *Pisum sativum*.

3. Candidate S8: identical to the *Arabidopsis thaliana* mRNA of cytochrome p450 (dbj accession number D78598.1; Mizutani *et al.*, unpublished). However, this candidate was inserted in false-orientation therefore codes for a peptide of 86 amino acids with no identity to any known proteins.

4. Candidate S38: identical to the coding sequence of the *Arabidopsis thaliana* putative cytosolic factor gene (gb accession number AY045913.1; Yamada *et al.*, unpublished). Because of a reading frame shift, it codes for a 12-amino acid peptide with no identity to known proteins.

5. Candidate S35: identical to the complement of the mRNA of *Arabidopsis thaliana* Glutamyl-tRNA synthetase (gb accession number is AF067773), encoding the N-terminal part (~261aa) of AtGluRS protein with an additional 20 amino acids appended to the N-terminus. This candidate is identical to pACT2-GluRS(-20-261) which emerged at the screen with pGBT9-HB6dC269.

The sequence analysis results suggested that the AtGluRS might interact with itself and form homodimers. And the interaction candidates encoding proteins with consensus to the senescence-associated protein ssa-13 implied that the AtGluRS might have some relations with senescence regulation even though no identical protein of ssa-13 has been found so far in *Arabidopsis*.

When AtGluRSfl was used as the bait protein to screen the 100 μg Ohio library DNA (total transformants was $5,6\times10^5$), 53 *His3* positive candidates were obtained, but none of them displayed *LacZ* positive. Thus, no *bona fide* interaction partner was obtained.

3.3.3 Homodimerization of AtGluRS in yeast

In wheat, three kinds of GluRS (cytoplasmic, chloroplast, and mitochondrial) were isolated as dimers and they acted as dimers in equilibrium with inactive monomers (Ratinaud *et al.*, 1983). In the above screen, AtGluRS(-20-261) emerged as an interacting partner of the cytoplasmic AtGluRS(1-261), indicating the homodimerization of AtGluRS in yeast. To identify the region responsible for homodimerization, the potential of interaction was tested in the yeast two-hybrid system with different versions of AtGluRS.

When AtGluRS(1-261) was used as DBD-fusion protein, the participation of AD-fusions AtGluRS(1-216), AtGluRS(1-261), or AtGluRS(1-445) could activate the *His3* reporter and the co-transformed yeast could grow well on the SD medium without histidine (Figure I-54). In agreement with this finding, the β-galactosidase quantitative assay showed that the combination of DBD-AtGluRS(1-261) and AD-AtGluRS(1-261) activated the *LacZ* gene quite efficiently, and the combinations of DBD-GluRS(1-261) and AD-AtGluRS(1-216) or AD-AtGluRS(1-445) could also slightly activate the *LacZ* gene and resulted in a β-galactosidase activity at 0.2-0.5 units. In view of the lack of both *His3* reporter and *LacZ* gene activation in the co-transformants, it was deduced that none of the other versions of AtGluRS were able to interact with AtGluRS(1-261). The region of 110-216aa is most likely critical for the interaction in yeast cells, and the portion (216-261aa) closely linked to this region may play a role in reinforcing the interaction, while the C-terminal part most likely negatively influence the interaction.

Using the full-length AtGluRS as bait, 0.2-0.5 units of β-galactosidase activity as well as the cell growth were perceived when the N-terminal domain of AtGluRS, the AtGluRS(1-261), AtGluRS(1-216) or AtGluRS(1-445), was combined as the AD fusion (Figure I-55). It indicates the weak interaction of full-length AtGluRS and these N-terminal versions of AtGluRS.

No activation was detected between DBD-AtGluRSfl and the other AD-AtGluRS versions (Figure I-55). To some degree, these results were consistent with those of using AtGluRS(1-261) as the DBD-fusion. However, what different is that, in this case, weak interactions of full-length AtGluRS with some AtGluRS versions were detected.
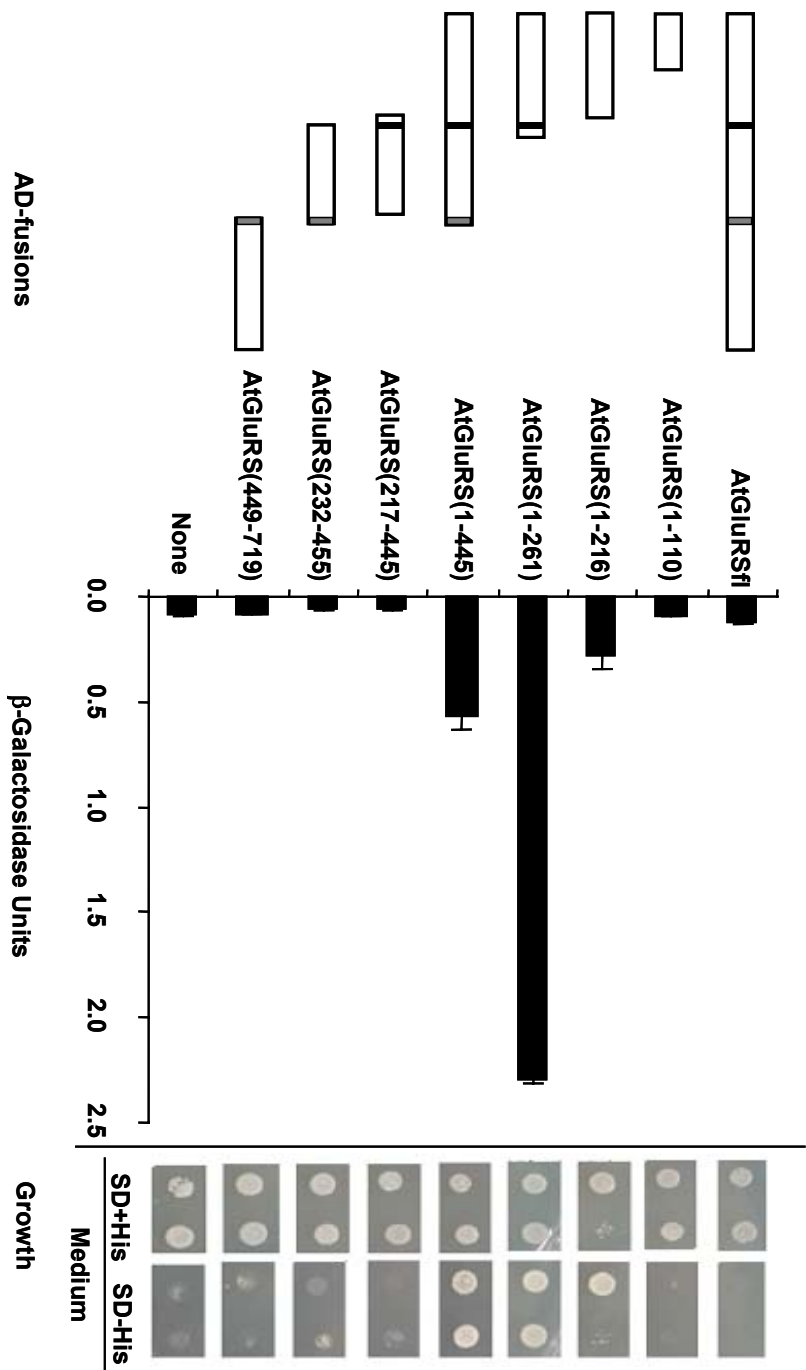
Figure I-54. Two-hybrid interactions of the bait AtGluRS(1-261) and different versions of prey AtGluRS. Left: different AtGluRS versions fused to AD. Middle: Quantitative measurement by β-galactosidase liquid assay; Right: Cell growth on SD selective medium with or without histidine.

AD-fusions

None
AtGluRS(449-719)
AtGluRS(232-455)
AtGluRS(217-445)
AtGluRS(1-445)
AtGluRS(1-261)
AtGluRS(1-216)
AtGluRS(1-110)
AtGluRSfl

β-Galactosidase Units

0.0   0.5   1.0   1.5   2.0   2.5

Growth

SD+His   SD-His
Medium
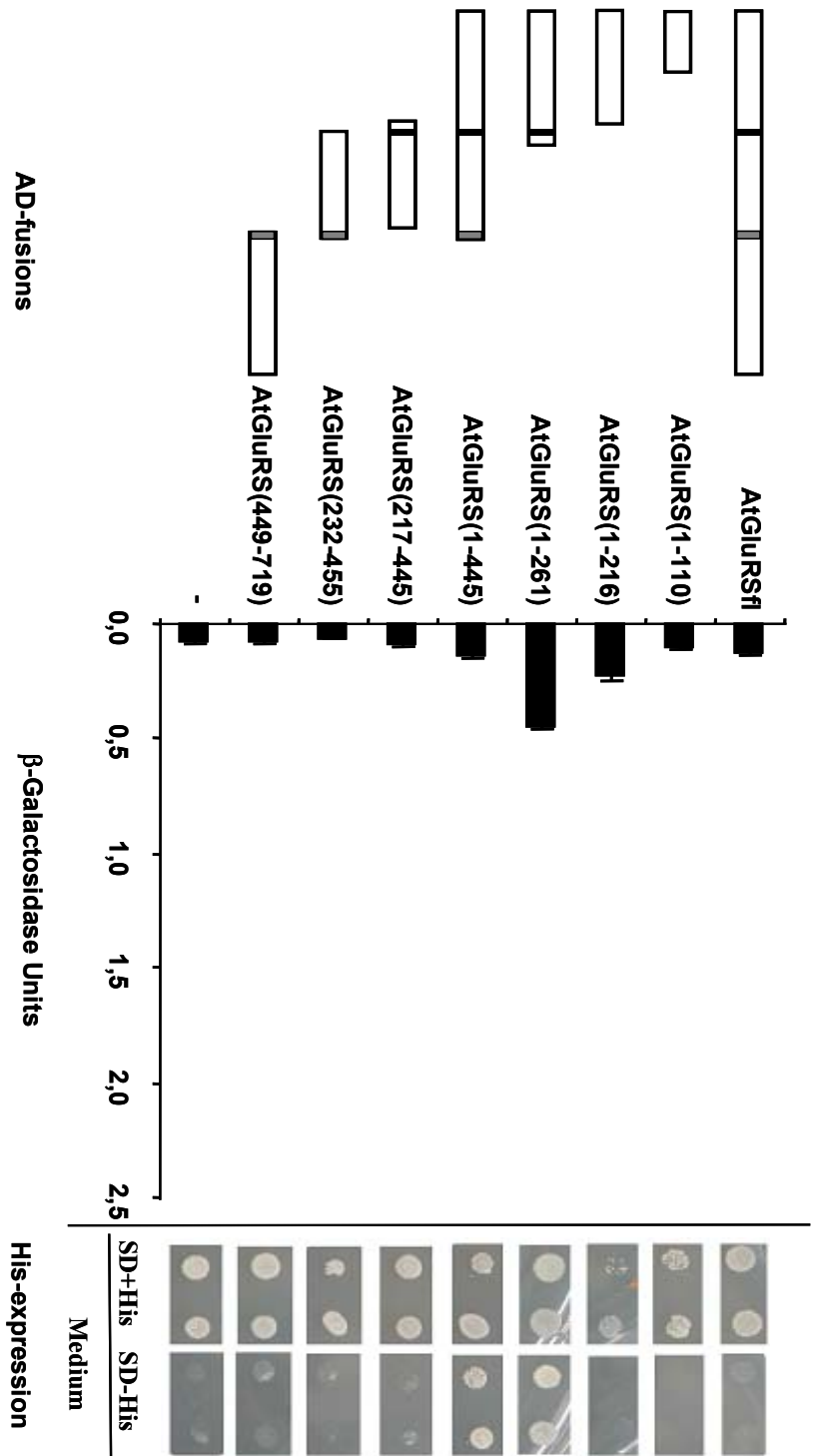
Figure I-55. Two-hybrid interactions of the bait AtGluRSfl and different versions of prey AtGluRS. Left: different versions of AtGluRS fused to AD. Middle: Quantitative measurement by β-galactosidase liquid assay; Right: Cell growth on SD selective medium with or without histidine.

3.4   Analysis of the function of *Arabidopsis* AtGluRS in yeast

3.4.1 Interactions of the full-length AtGluRS with other proteins in yeast

The above results indicate that AtGluRS interacts with AtHB6, AtHB7 or ABI1 in yeast only as a short truncated version (AtGluRS(1-261)). The apparent lack of physical interaction between the full-length AtGluRS and any other proteins tested was surprising.

There are three possible explanations for this phenomenon.

1. Even though the *bona fide* interactions of full-length AtGluRS with its partners occur, it cannot import the nucleus to activate the reporter genes because of the large molecule size of AtGluRS or the complex. To test this possibility, the DBD-HB7fl fusion, which could strongly auto-activate the *LacZ* gene without AD-partner (Figure I-56), was combined with AD-GluRSfl in the yeast two-hybrid system. If the conjecture was correct, retaining the protein complex AtHB7-AtGluRS in the cytoplasm would reduce the amount of DBD-AtHB7fl imported into the nucleus. Consequently, the activation of the reporter gene induced by DBD-AtHB7fl would be decreased. However, neither AtGluRS(1-261) nor full-length AtGluRS are able to reduce the expression of the reporter gene activated by DBD-AtHB7 (Figure I-56).
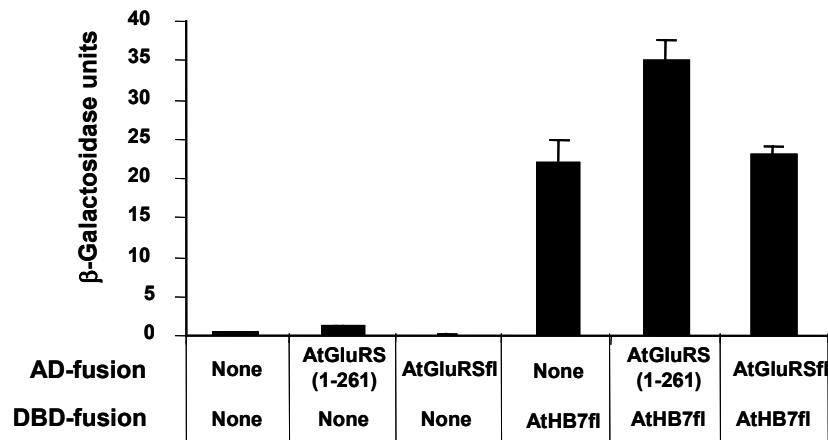
Figure I-56. AtGluRS fusion does not retain AtHB7 from the nucleus. Yeast strain HF7c was co-transformed with pACT2-GluRSfl or pACT2-GluRS(1-261) as prey and pGBT9-HB7fl as bait. The empty vector pACT2 and pGBT9 were used as the non-fusion protein control. β-galactosidase activity was determined in three independent colonies and no reduction of the auto-activation of AtHB7 by either AtGluRSfl or AtGluRS(1-261) was shown.

Figure I-57. Comparison of GluRS amino acid sequences of *S. cerevisiae* (1), *A. thaliana* (2), *M. musculus* (3) and *E. coli* (4). The conserved amino acids of the domains in yeast GluRS which are responsible for complex formation are marked out by underline. The putative ATP binding site "HIGH" motif and the "LSKRK" motif at the putative binding site of the 3'end of tRNA are marked by two boxes. Sequences aligned score (%) are: (1:2) – 43; (1:3) – 20; (1:4) – 13; (2:3) – 21; (2:4) – 13; (3:4) – 13.

```
        1        10        20        30        40        50        60        70        80        90
        I---------+---------+---------+---------+---------+---------+---------+---------+---------I
    1                          MTKLFSKVKESIEGIKMPSTLTINGKAPIVAYAELIAARIVNALAPNSIAIKLVDDKKAPAAKLD
    2                            MDGMKLSFPPESPPLSVIVALSLSASPVTIDSSAAATTVPSFVFSDGRKLNGAT
    3    MATPDSLALFTGLGLSENKARETLKNEALSTQLREAATQAHQILGSTIDKATGVLLYDLV--SRLRDTRRRSFLVSYIANKKIHTGLQLS
    4
  Cons.  ..............................................................................................

        91       100       110       120       130       140       150       160       170       180
        I---------+---------+---------+---------+---------+---------+---------+---------+---------I
    1    DATEDVFNKITSKF--AAIFDNG--DKEQVAKWVNLAQKELVIKNFAKLSQSLETLDSQL---NLRTFILGGLKYSAADVACWGALRSNG
    2    VLLRYV-GRSAKKL--PDFYGNNAFDSSQIDEWVDYAS---VFSSGSEFENACGRVDKYL---ESSTFLVGH-SLSIADVAIWSALAGTG
    3    AALEYVRSHPQDPIDTKDFEQECGVGVVVTPEQIEEAVESTINKHQLQLLAERYRFNMGLLMGEARAALRWADGKMIKNEVDMQVLHLLG
    4
  Cons.  .....v...............................a.....................l....................l...g

        181      190       200       210       220       230       240       250       260       270
        I---------+---------+---------+---------+---------+---------+---------+---------+---------I
    1    M-CGSIIKNKVDVNVSRWYTLL--EMDPIF--GEAHDFLSK-------SLLELKKSANVGKKKETHKANFEIDLPDAKM----GEVVTRF
    2    QRWESLRKSKKYQSLVRWFNSILDEYSEVL--NKVLATYVKKGSGKPVAAPKSKDSQQAVKGDGQDKGKPEVDLPEAEI----GKVKLRF
    3    PKMEADLVKKPKVAKARLEETDRKTAKDVVEKGEVAGQTLSLMEQLRGEALKFHKPGENYKTPGYVITPYTMDLLKQHLEITGGQVRTRF
    4                                                                              MKIKTRF
  Cons.  ..........k......r.....................................k.........dl.........gk!ktRF

        271      280       290       300       310       320       330       340       350       360
        I---------+---------+---------+---------+---------+---------+---------+---------+---------I
    1    PPEPSGYLHIGHAKRAALLNQYFAQAYKGKLIIRFDDTNPSKEKEEFQDSILEDLDLLGIKGDRITY-SSDYFQEMYDYCVQMIKDGKAYC
    2    APEPSGYLHIGHAKRALLNKYFAERYQGEVIVRFDDTNPAKESNEFVDNLVKDIGTLGIKYEKVTY-TSDYFPELMDMAEKLMREGKAYV
    3    PPEPNGILHIGHAKAINFNFGYAKANNGICFLRFDDTNPEKEEAKFFTAIYDMVTWLGYTPYKVTY-ASDYFDQLYAWAVELIHGGLAYV
    4    APSPTGYLHVGGARTALYSWLFARNHGGEFVLRIEDTDLERSTPEAIEAIMDGMNWLSLEWDEGPYYQTKRFDRYNAVIDQMLEEGTAYK
  Cons.  aPeP.GyLH!GhAkaal.n..%A....Ge..lRf#DT#peke..ef..ai.d...wLg...d..tY..sdyFd..a....q$..eG.AY.

        361      370       380       390       400       410       420       430       440       450
        I---------+---------+---------+---------+---------+---------+---------+---------+---------I
    1    DDTPTEKMREERMDGVASARRDRSVEENLRIFTEEMKNGTEEGLKNCVRAKIDYKALNKTLRDPVIYRCNLTPHHRTGSTWKIYPTYDFC
    2    DDTPREQMQKERMDGIDSKCRNHSVEENLKLW-KEMIAGSERGLQCCVRGKFNMQDPNKAMRDPVYYRCNPMSHHRIGDKYKIYPTYDFA
    3    CHQRVEELKGH--NPLPSPWRDRPKEESLLLF-EAMRKGKFAEGEATLRMKLVMED---GKMDPVAYRVKYTPHHRTGDKWCIYPTYDYT
    4    CYCSKERLEALREEQMAKG--EKPRYDGRCRHSHEHHADDEPCVVRFANPQEGSVYFDDQIRGPIEFS-NQELDDLIIRRTDGSPTYNFC
  Cons.  :....E.$....r.#...as..r#.p.e#.l.....em.ag.e........r.k...........rdP!.%r.n...hhrig....iyPTY#%c

        451      460       470       480       490       500       510       520       530       540
        I---------+---------+---------+---------+---------+---------+---------+---------+---------I
    1    VPIVDAIEGVTHALRTIEYRDRNAQYDWHLQALRLRKVHIWDFARINFVR-TLLSKRKLQWHVDKDLVGNWDDPRFPTYRGVR------R
    2    CPFVDSLEGITHALRSSEYHDRNAQYFKVLEDMGLRQVQLYEFSRLNLVF-TLLSKRKLLWFVQTGLVDGWDDPRFPTYQGIV------R
    3    HCLCDSIEHITHSLCTKEFQARRSSYFWLCNALKVYCPVQWEYGRLNLHY-AVVSKRKILQLVAAGAVRDHDDPRLFTLTALR------R
    4    VVYDDWDMEITHVIRGEDHINNTPRQINILKALKAPVPVYAHVSMINGDDGKKLSKRHGAVSVMQYRDDGYLPEALLNYLVRLGWSHGDQ
  Cons.  ,....D..e.!TH.lr..#....r...y....l.a$k...pv....sriN.......lSKRk....V.....vdgwddprl.t............r

        541      550       560       570       580       590       600       610       620       630
        I---------+---------+---------+---------+---------+---------+---------+---------+---------I
    1    RGMTVEGLRNFVLSQGPSRNVINLEWNLIWAFNKKVIDPIAPRHTAIVNPVKIHLEGSEAPQEPKIEMKPKHKKNPAVGEKKVIYYKDIV
    2    RGLKIEALIQFILEQGASKNLNLMEWDKLWSINKRIIDPVCPRHTAVYAERRVLFTLTDGPDEPFVRHIPKHKKFEGAGEKATTFTKSIW
    3    RGFPPEAINNFCARVGVTVAQTTHEPHLLEACVRDVLNDAAPRAMAVLEPLQVVITNFPAPKPLDIRV-PNFPADETKGFHQVPFASTVF
    4    EIFTREEMIKYFTLNAVSKSASAFNTDKLLWLNHHYINALPPEYVATHLQWHIEQENIDTRNGPQLADLVKLLGERCKTLKEMAQSCRYF
  Cons.  -gft.E..i.%....gvsk.....#.dkl...n...i#...Pr..A......!..en.d.p..p......pk......kg.k.........f

        631      640       650       660       670       680       690       700       710       720
        I---------+---------+---------+---------+---------+---------+---------+---------+---------I
    1    VDK-DDADV---------INVDEEVTLMDWGNVI-ITK--KNDDGSMVAK---LNLEGDFKKTKHKLTWLADTKDV-YPVDLVDFDHLITK
    2    LEE-ADASA---------ISVGEEVTLMDWGNAI-VKEITKDEEGRVTALSGVLNLQGSVKTTKLKLTWLPDTNEL-VNLTLTEFDYLITK
    3    IER-SDFKEESEPGYKRLASGQPVGLRHTGYVIELQNIVRGSSGCVERLEVTCRRADAGEKPKAFIHWVSQPLVCEIRLYECLFQHKNPE
    4    YEDFAEFDADAAKKHLRPVARQPLEVVYRDKLTAITDWTAENVHHAIQATADELEVGHGKVGHPLRVAVTGAGQSPALDVTYHAIGKTRSI
  Cons.  .#..a#fda.......r....#pv.l...g..i.......n..g...a....l...........kl...w.........vt...f......

        721      730       740       750       760       770       780       790      798
        I---------+---------+---------+---------+---------+---------+---------+----------+-------I
    1    DRLEEDESFEDFLTPQT-EFHTDAIADLNVKDMKIGDIIQFERKGYYRLDA-LPKDGKPYVFFTIPDGKSVNKYGAKK
    2    KKLEDDDEVADFVNPNT-KKETLALGDSNMRNLKCGDVIQLERKGYFRCDVPFVKSSKPIVLFSIPDGRAAK
    3    DPVEVPGGFLSDLNPASLQVVEGALVDCSVALAKPFDKFQFERLGYFSVD-PDSHQGQIVFNRTVTLKEDPGKI
    4    ERINKALAFIAERENQQ
  Cons.  .r.#....f......pq........a..d......k..d..q.er.gy...d...........................
```

2. The AtGluRS behaves similarly to the yeast GluRS. It forms complexes with other yeast proteins (*e.g.* MetRS, Arc1p) in the cytoplasm, thus leading to its exclusion from the nucleus (Galani *et al.*, 2001) and no activation of the reporter gene. However, the presumptive region required for the GluRS-MetRS-Arc1p complex formation is the residues 87-170 in yeast GluRS (Galani *et al.*, 2001), corresponding to residues 76-156 in AtGluRS (Figure I-57). And the N-terminal version AtGluRS(1-261) contains this region, but still could strongly activate the reporter gene when combined with the other fusion proteins (*e.g.* AtHB6, ABI1). It indicates that the AtGluRS(1-261) as well as AtGluRSfl is not excluded out of nucleus as the presumed complex formation with MetRS and Arc1p.

3. The full-length AtGluRS does not function in the protein interaction because of improper folding or steric hindrance yeast. This would be the most acceptable explanation. Thus, the function of AtGluRS was further assayed.

3.4.2 The potential of *Arabidopsis* AtGluRS to substitute for yeast AtGluRS

It was reported that *E. coli* tyrosine tRNA synthetase could substitute the corresponding yeast mitochondrial enzyme in function, although the yeast mitochondrial and the *E. coli* tyrosine tRNA synthetase have much differences in sequence (Edwards and Schimmel, 1987). When comparing the AtGluRS with the GluRS from yeast, mouse and *E. coli*, the closest homology (43% identity) is found with the yeast GluRS (Figure I-57). The region of highest homology extents from the 'HIGH' motif to the C-terminal end of the protein. This region forms the catalytic domain. The homology indicates that the *Arabidopsis* AtGluRS may substitute the yeast GluRS in function.

To study the ability of *Arabidopsis* AtGluRS to complement yeast deficient in GluRS, AtGluRS expressed from the constructs pACT2-GluRSfl and pBridge (with NLS deletion)//GluRSfl were used to complement the yeast *gluRS* null strain (Galani *et al.*, 2001). As controls, pRS315 and pRS315-yGluRS (yeast GluRS) were used.
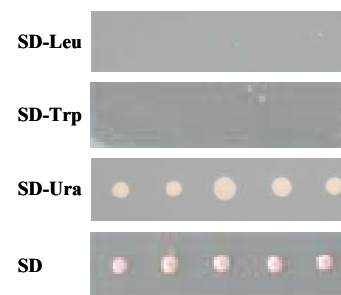

Figure I-58. The ability of yeast strain 1605# to grow on different selective medium.

Yeast strain and constructs

The yeast strain 1605# and plasmids pRS315 and pRS315-yGluRS (Galani *et al*., 2001) were kindly provided by Professor Simos of the Biochemie-Zentrum Heidelberg. The

yeast strain 1605# is a *gluRS* null strain carrying pRS316-yGluRS to complement the mutation of the GluRS gene. In addition to the mutation in the GluRS gene, the host strain is auxotrophic for *Ura, Leu, Trp, His* and *Ade* (Figure I-58). pRS316-yGluRS carries the *URA* gene, and both of the control plasmids pRS315 and pRS315-yGluRS carry the nutritional *LEU* marker gene.

pACT2 is a vector from the yeast two-hybrid system that generates an AD-fusion protein which can be imported into the nucleus directed by the SV40 large T-antigen nuclear localization signal (NLS). This plasmid carries the *LEU2* gene for selection of *Leu* auxotrophic yeast strains. pACT2-GluRSfl generates NLS-AD-GluRS protein. pBridge is a vector from the yeast three-hybrid system carrying the *TRP1* gene to allow selection of yeast strains which are deficient in tryptophan biosynthesis. In order to prevent AtGluRS import into the nucleus directed by the NLS provided for the DBD-fusion, the pBridge was cut by *Nde*I (filled in) and *Bgl*II to delete the NLS region linked immediately to the *MSCII*. The full-length AtGluRS cDNA obtained from pGEX-GluRS by *EcoR*I partial digestion (filled in) and subsequent *Bgl*II digestion was cloned into this site to construct the pBridge(dNLS)//GluRSfl (Figure I-59) and this construct was sequenced to make sure the cDNA is in correct reading frame.
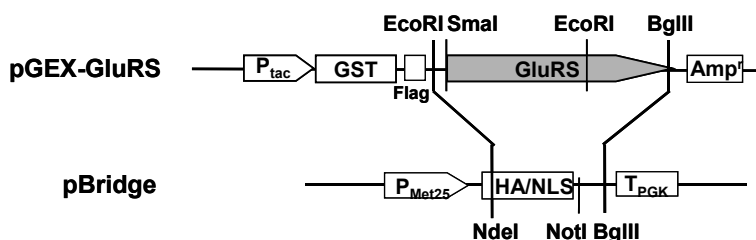
Figure I-59. Construction of pBridge (dNLS)//GluRSfl.

Complementation analysis of yeast *gluRS⁻* by *Arabidopsis* AtGluRS

Different plasmids carrying AtGluRS genes (or not) were introduced into strain 1605#. Cultivation for 3 days (approxi. 30 generations) in medium supplement with uracil generated none selective conditions to allow loss of the pRS316-yGluRS. The probability of loss of pRS316-yGluRS was surveyed afterwards by cultivating the yeast colonies on medium selecting for both pRS316-yGluRS and the newly introduced plasmid. If the newly introduced plasmid was able to complement the *gluRS⁻*, the cells would be able to lose pRS316-yGluRS. And loss of pRS316-yGluRS resulted in uracil auxotrophy and cessation of growth on medium-Ura. Those non-selective plasmids are

frequently lost unless there exist a selective pressure for maintenance (Galani *et al.*, 2001).

The yeast 1605# cells transformed with pRS315-yGluRS as the positive control or with pRS315 as the negative control were cultivated in the liquid SD medium without leucine. The cultures were diluted in 1:1000 fresh medium every day and after 30 generations cultivation, the cells were spread on SD-Leu plate to obtain single colonies. 40 to 96 single colonies were picked for testing growth on SD-Leu-Ura plates to determine the probability of loss of pRS316-yGluRS. Figure I-60A shows that 56% colonies of the pRS315-yGluRS -transformed yeast cells lost pRS316-yGluRS but had pRS315-yGluRS. As predicted, the empty vector pRS315 could not substitute pRS316-yGluRS and pRS316-yGluRS was not lost due to the selective pressure for a functional GluRS gene (Figure I-60B). However, when the pACT2-GluRSfl -transformed yeast 1605# cells were grown in SD-Leu for 30 generations of plasmid-lossing cultivation, all selected colonies could grow on SD-Leu-Ura as well as on SD-Leu plates (Figure I-61), indicating that none of the colonies lost the *URA*-carrying plasmid pRS316-yGluRS. Considering that AtGluRS expressed in pACT2-GluRSfl is fused with an NLS, probably the import of AtGluRS to the nucleus directed by this NLS restrained the function of AtGluRS and led to the failure of substitution. pBridge(dNLS)//GluRS was further transformed to the yeast 1605#. However, it was still unable to cause the loss of the plasmid pRS316-yGluRS after 30 generations cultivation in SD-Trp selective medium (Figure I-62).

It should be noted that sequencing of AtGluRS(2.2Kb) in pBridge(dNLS)//GluRS and pACT2-GluRSfl led to the discovery of 2 changed amino acids as compared with the *Arabidopsis* Columbia ecotype AtGluRS protein sequence in databank. Those changes are Val (580) to Ile, and Asp (654) to Glu (Figure I-63). It is not clear if these changes are due to the differences existing between ecotype Columbia and RLD, the later one is our AtGluRS generated from (Yang, 2003). However, these different amino acids are conservative and probably do not interfere with protein function.

In conclusion, even though *Arabidopsis* AtGluRS and yeast GluRS show high consensus (43%), the AtGluRS was not able to substitute the yeast *gluRS⁻* either in the form with NLS (pACT2-GluRSfl) or without NLS (pBridge(dNLS)//GluRSfl). Probably the AtGluRS cannot be folded properly in the foreign environment in yeast (Aloy and Russell, 2002) therefore it fails to function well. This may be the reason of that no interaction was detected between full-length AtGluRS with ABI1, AtHB6 and AtHB7 in yeast system.

SD-Leu   SD-Leu-Ura

A

0 generation

30 generations

B

0 generation

30 generations
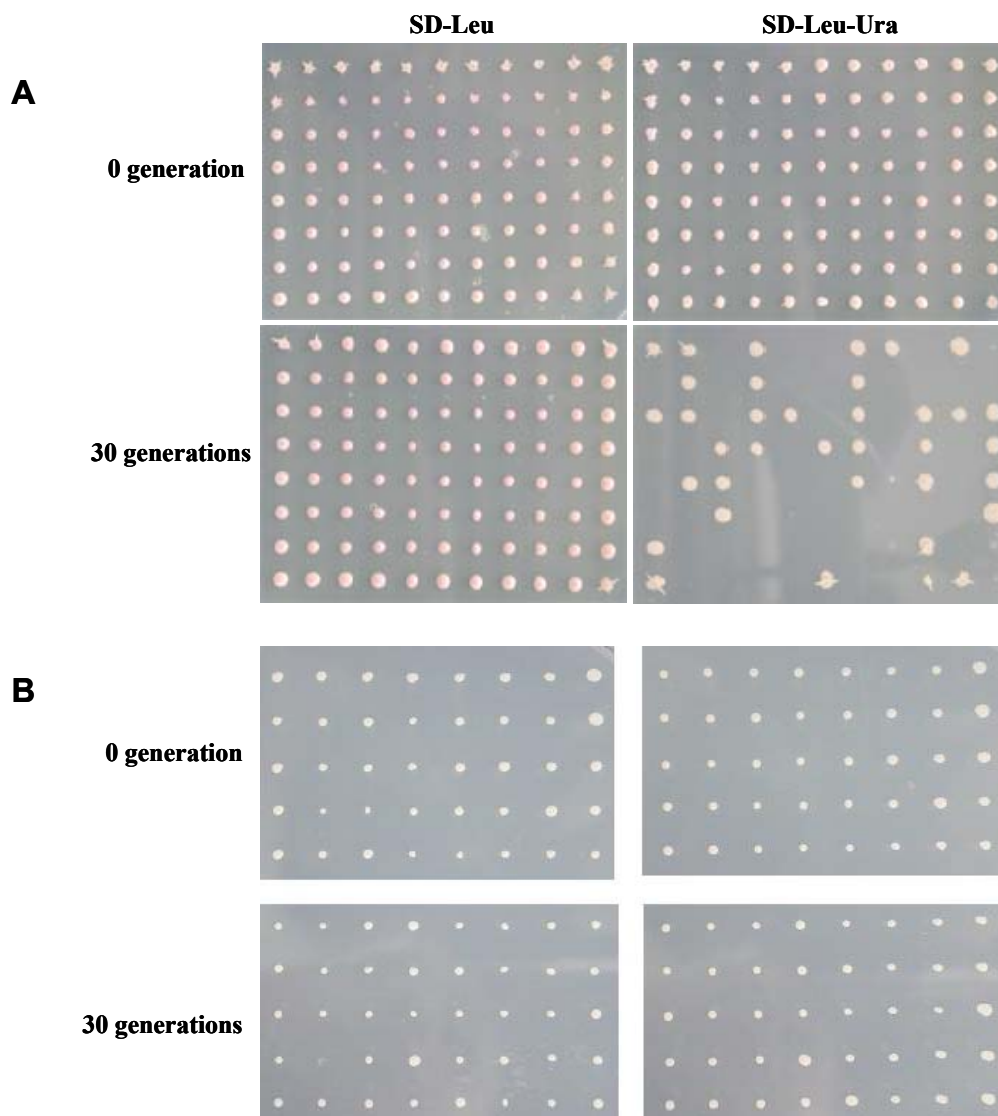
Figure I-60. Complementation assay of yeast mutant *gluRS⁻* by pRS315-yGluRS. Yeast strain 1605# harboring the plasmid pRS316-yGluRS providing Ura-autotrophy was transformed with pRS315-yGluRS (A) or with empty plasmid pRS315 (B) providing Leu-autotrophy, and subsequently cultivated in non-selective medium for 0 or 30 generations prior analysis of growth on selective medium SD–Leu or –Leu-Ura. Successful complementation had been occurred in the colonies those could grow on SD-Leu but not on SD-Leu-Ura.
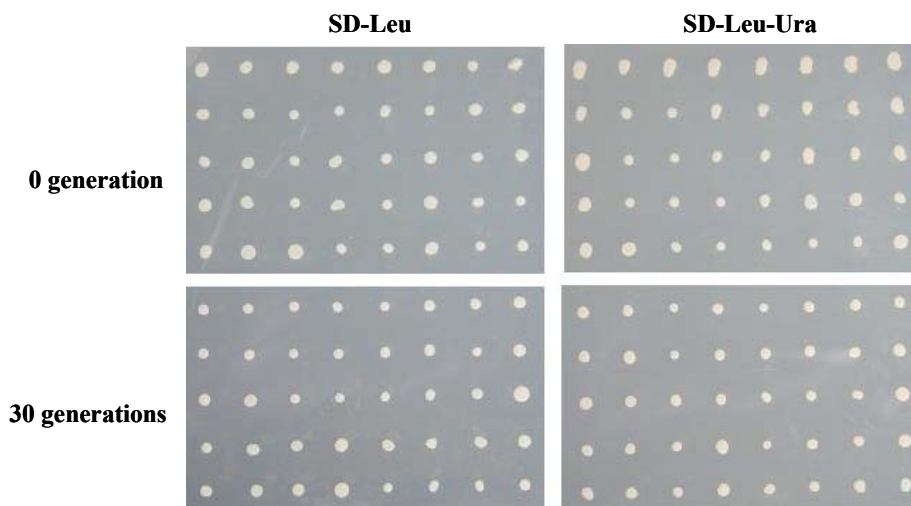
Figure I-61. Complementation assay of yeast mutant *gluRS⁻* by *Arabidopsis* AtGluRS expressed from pACT2-GluRS. Yeast strain 1605# harboring the plasmid pRS316-yGluRS providing Ura-autotrophy was transformed with pACT2-GluRS providing Leu-autotrophy and subsequently cultivated in non-selective medium for 0 or 30 generations prior analysis of growth on selective medium SD–Leu or –Leu-Ura.
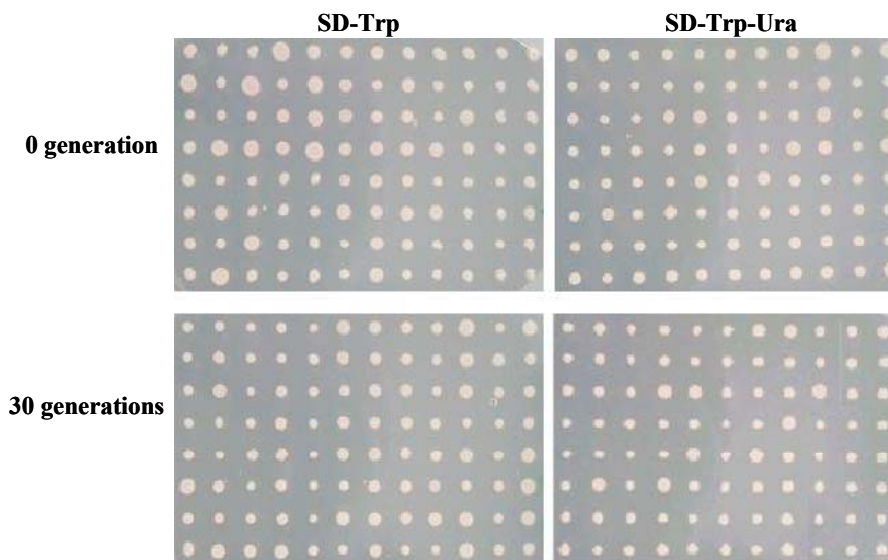
Figure I-62. Complementation assay of yeast mutant *gluRS⁻* by *Arabidopsis* AtGluRS expressed from pBridge(dNLS)//GluRS. Yeast strain 1605# harboring the plasmid pRS316-yGluRS providing Ura-autotrophy was transformed with pBridge(dNLS)//GluRS providing Trp-autotrophy and subsequently cultivated in non-selective medium for 0 or 30 generations prior analysis of growth on selective medium SD–Trp or –Trp-Ura.

Figure I-63. Comparison of the sequence of AtGluRS in pACT2 with AtGluRS in data bank. The mutations in pACT2-GluRS were marked out by a rectangle.
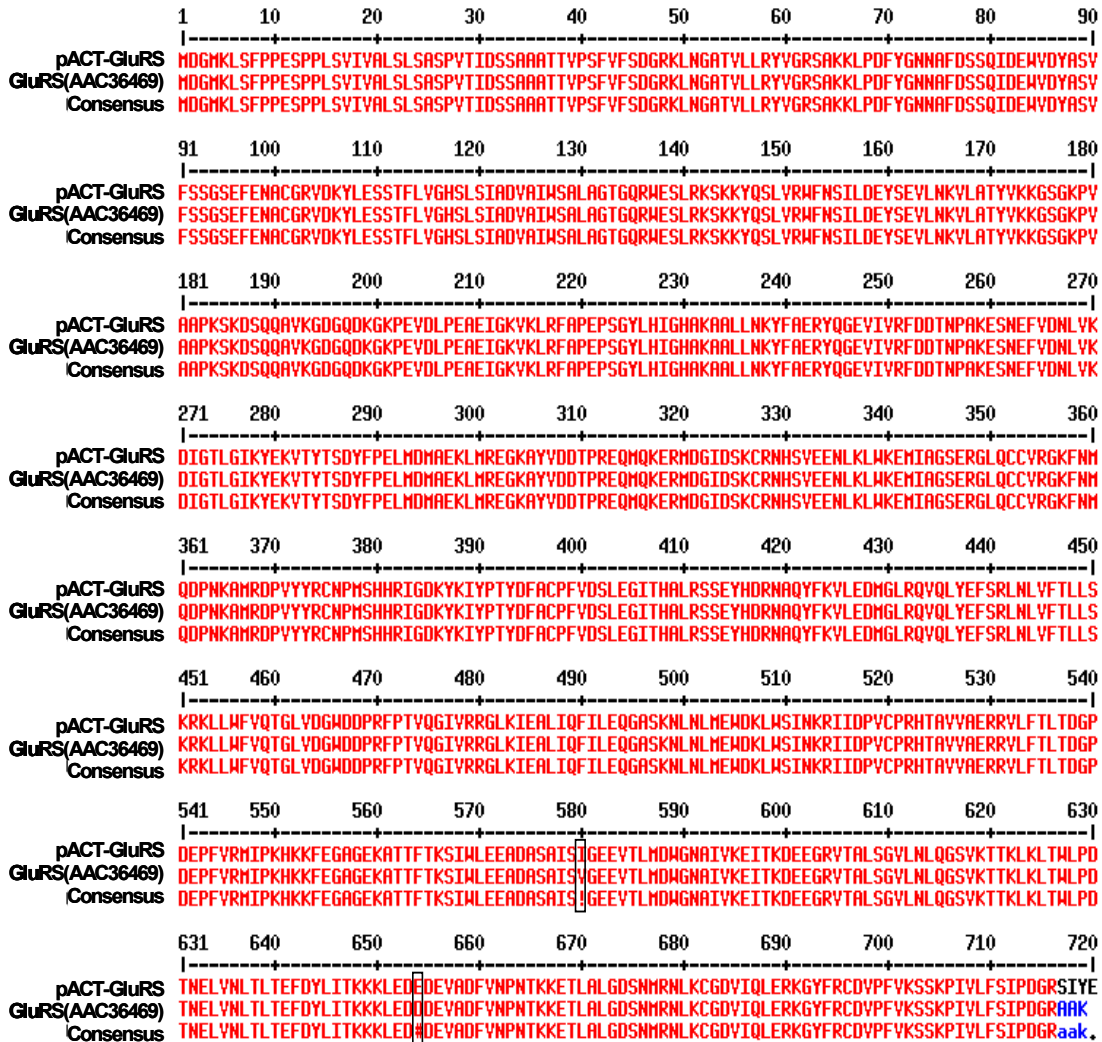
3.5   Effect of AtGluRS on the cellular localization of AtHB6

3.5.1   Cellular localization of AtGluRS

The cytoplasmic GluRS in yeast has been reported to localize either in the cytoplasm, or in the nucleus when its assembly into a multi protein complex is inhibited (Galani *et al.* 2001). The results of the yeast two-hybrid analysis revealed an interaction of AtGluRS with AtHB6, AtHB7, ABI1 and ABI2. Results from the *in vitro* pull down assay and *in*

vivo co-immunoprecipitation experiment argue for a physical interaction of AtGluRS with ABI1 and abi1. However, AtHB6 was reported to localize in the nuclear compartment (Himmelbach *et al.*, 2002) and ABI1 also has a monopartite nuclear motif (KPRRK) targeted at its C-termini (423-427 residues) and has been localized in nucleus (Himmelbach, personal communication).

Recently, the *Agrobacterium*-transformed plant cells system has been utilized successfully for epitope-tagged protein detection (Ferrando *et al.*, 2000; 2001). In that system, an intron *IV2* from the potato gene *ST-LS1* was inserted into the coding domain of HA or cMyc epitope. This allows the labeling of plant protein with an epitope-tag that is not expressed in *Agrobacterium*, and thus avoids the interference of expression of plant proteins in *Agrobacterium e.g.* in transient expression analyses (Ferrando *et al.*, 2000; 2001). To detect the cellular localization of the AtGluRS fusion protein, the pPCV-MESHI-GluRS-GUS was constructed as follows: the β-glucuronidase (GUS) cDNA (1.88 kb) fragment was obtained by digestion of pBI221-GUS with *BamH*I and *EcoR*I, and then cloned into the *BamH*I and *EcoR*I sites C-terminally to the intron-disrupted cMyc epitope of plasmid pMESHI to construct pMESHI-GUS. The *Sma*I-*Bgl*II fragment of full-length AtGluRS cDNA from pGEX-GluRS was subsequently inserted into the *Bgl*II and filled-in *Xba*I sites N-terminally to the cMyc epitope of pMESHI-GUS (Figure I-64). To generate pPCV-MESHI-GluRS-GUS, the expression cassette was moved from pMESHI-GluRS-GUS by *Not*I into the binary vector pPCV812-MENSHU with the third previously destroyed *Not*I site which is far away from expression cassette (Figure I-64).

The pPCV-MESHI-GluRS-GUS, or the pPCV812-GIGI (Ferrando *et al.*, 2000) and pPCV812-MESHI-GUS, as the negative and positive controls, were transformed into *Agrobacterium* GV3101 (pMP90RK). Five days after infection by these *Agrobacterium*, the transformed *Arabidopsis* cells were subjected to GUS staining. Counterstaining of nuclei with DAPI was used to identify the location of the nuclei. The non-fused GUS protein was found distributed in the whole cell. The AtGluRS-GUS fusion protein was strictly localized in cytoplasm and exclusively out of the nucleus as specks. It is not clear whether these specks are due to organelles or other subcellular structures like protein complexes (Figure I-65).

3.5.2 AtGluRS alters the cellular localization of AtHB6

Since AtGluRS was presumably localized in the cytoplasm (Figure I-65), and AtHB6 was reported to be localized in the nuclear compartment (Himmelbach *et al.*, 2002), the interesting question is how can AtGluRS and AtHB6 interact despite different cellular compartments? To address it, the *Arabidopsis* transgenic lines with overexpression of
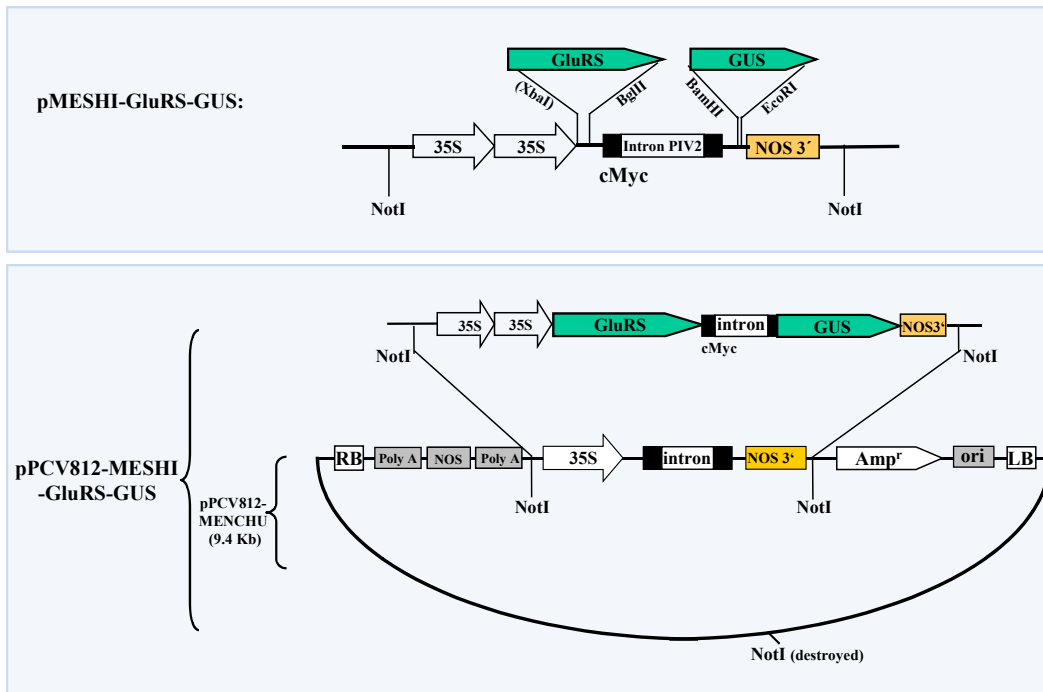
Figure I-64. Constructs of pMESHI-GluRS-GUS and pPCV812- pMESHI-GluRS-GUS. The cloning sites are labelled. LB and RB represent the left and right borders of T-DNA. The third *Not*I site was destroyed by filled-in and re-ligation.

Figure I-65. Cellular localization of AtGluRS fused to GUS. The suspension cells of *Arabidopsis* were transformed with AtGluRS-GUS fusion construct and were stained for both GUS activity and DNA by DAPI to reveal the nucleus (indicated by arrow). The cells transformed with the empty vector and with GUS construct were used as control. The AtGluRS-GUS fusion protein is shown to be strictly cytosolic localization.

the fusion protein AtHB6-GUS (Himmelbach *et al.*, 2002) were crossed to AtGluRS overexpressing sense transgenic lines (GS), to AtGluRS-antisense line (GAS; Yang, 2003), or to wild type (RLD). As the control for the AtHB6-GUS line, a GUS-overexpression line (GUS) was used. Since the AtHB6 was reported to be localized in roots (Himmelbach *et al.*, 2002) as well as in stomata cells in developing cotyledons and the vascular tissue (Soderman *et al.*, 1999), 5 days old F1 seedlings and leaf epidermis of 3 weeks old F1 plants were subjected to histological analysis for GUS activity. Figure I-66 and Figure I-67 show the GUS staining and DAPI staining of root segments and single root cells of F1 seedlings, respectively. GUS activity in F1 from crosses of AtHB6-GUS to GAS or RLD was detected in the nucleus as expected, while GUS activity in F1 from crosses of AtHB6-GUS to GS3 (line 3) or to GS4 (line 4) was detected not only in the nucleus but also clearly in the cytoplasm. The analysis for GUS activity in mesophyll cells of cotyledons also demonstrated that the GUS activity in F1 from crosses of AtHB6-GUS to RLD was clearly distributed in nucleus and from crosses of AtHB6-GUS to GS4 was in whole cell (Figure I-68). Furthermore, the localization of GUS activity in guard cells was similar to that in root cells: Nuclear localization was detected in the F1 from crosses of AtHB6-GUS to RLD, and cytoplasmic localization was found in the F1 from crosses of AtHB6-GUS to GS3 (line 3) or GS4 (line 4) (Figure I-68). However, the GUS activity in F1 from crosses of AtHB6-GUS to GAS was also detected in the whole guard cell (Figure I-69), this is at variance to the situation observed in root cells. As controls, GUS expression in F1 of GUS-line crossed with GS, GAS or RLD distributed in the whole cell of either the root cells or the guard cells (Figure I-66; Figure I-67; Figure I-69). No effect of AtGluRS expression on the distribution of GUS protein was found. The results imply that the over-expression of AtGluRS alters the cellular distribution of AtHB6 partially from nucleus to cytoplasm.

| X-Gluc | DAPI | | X-Gluc | DAPI |
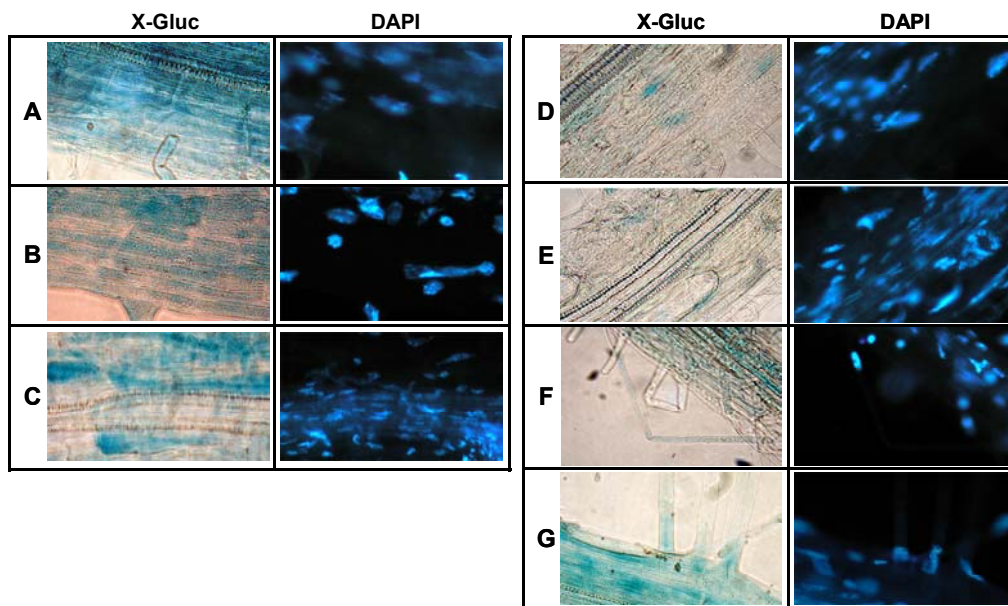
Figure I-66. Cellular localization of AtHB6 fused to β-glucuronidase affected by AtGluRS expression. F1 seedlings from different crosses were stained for both GUS activity by X-Gluc and for DNA to reveal the nucleus by DAPI. The staining indicative for GUS activity in root segments was photographed. A, B, C indicate the crosses of GUS overexpressing plant with RLD, GAS and GS4, respectively. D, E, F, G indicate the crosses of AtHB6-GUS overexpressing plant with RLD, GAS, GS4, GS3, respectively.

	X-Gluc	DAPI
RLDx GUS		
GAS x GUS		
GS4 x GUS		
RLD x AtHB6-GUS		
GAS x AtHB6-GUS		
GS3 x AtHB6-GUS		
GS4 x AtHB6-GUS		
GS3 x AtHB6-GUS		

Figure I-67. Cellular localization of AtHB6 fused to β-glucuronidase affected by AtGluRS expression. Seedlings from crosses as indicated were stained for both GUS activity by X-Gluc and for DNA to reveal the nucleus by DAPI. The staining indicative for GUS activity in single cells of root cortex was photographed.

	A few mesophyll cells		Single mesophyll cell	
	X-Gluc	**DAPI**	**X-Gluc**	**DAPI**
RLD x AtHB6-GUS				
GS4 x AtHB6-GUS				
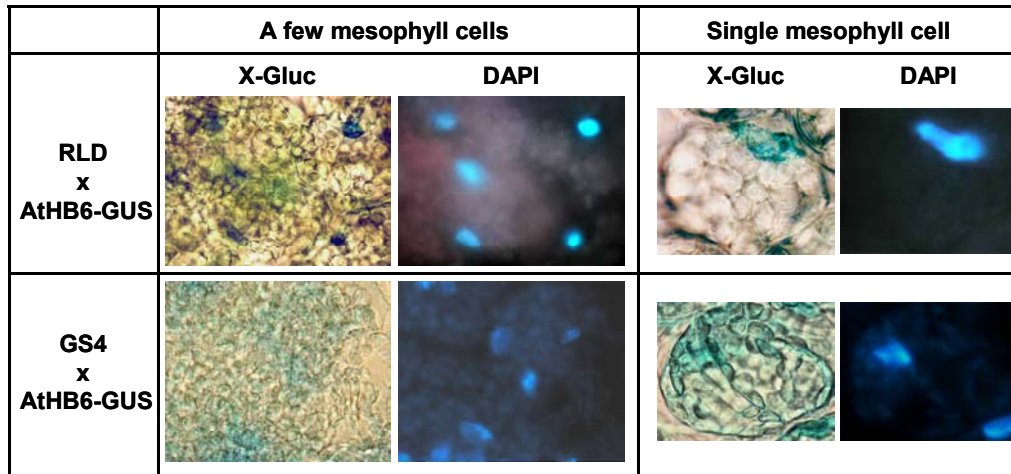
Figure I-68. Cellular localization of AtHB6 fused to β-glucuronidase affected by AtGluRS expression. *Arabidopsis* plants with over-expression of AtHB6-glucuronidase fusion protein (AtHB6-GUS) were crossed to plants with over-expression of AtGluRS (sense, GS) or RLD as the control. F1 seedlings were stained for both GUS activity by X-Gluc and for DNA to reveal the nucleus by DAPI. The staining indicative for GUS activity in mesophyll cells of cotyledons was photographed.
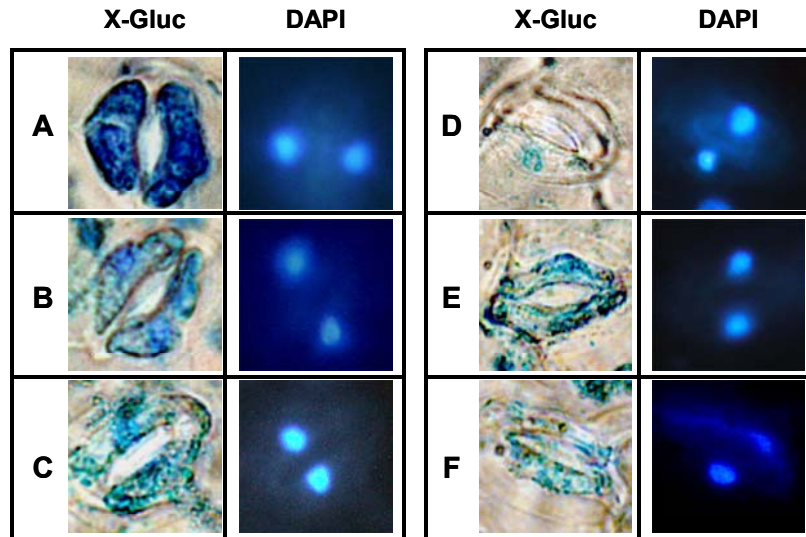
Figure I-69. Cellular localization of AtHB6 fused to β-glucuronidase affected by AtGluRS expression. F1 seedlings from different crosses were stained for both GUS activity by X-Gluc and for DNA to reveal the nucleus by DAPI. The staining indicative for GUS activity in guard cells were photographed. A, B, C indicate the crosses of GUS overexpressing plant with RLD, GS4 and GAS, respectively. D, E, F indicate the crosses of AtHB6-GUS overexpressing plant with RLD, GS4, GAS, respectively.

3.6 Regulation of *Rab18* transcription in transient expression system by ABI1, AtHB6 and AtGluRS

The expression of genes driven by different promoters such as *RD29B* (Yamaguchi-Shinozaki and Shinozaki, 1994), *Lti65* (Nordin et al, 1993), *Rab18* (Lang and Palva, 1992), and *AtHB6* (Soderman, 1999; Himmelbach *et al.*, 2002) are induced by ABA. Here, the negative ABA signaling regulators, ABI1 and AtHB6, as well as their interaction partners AtGluRS and AtHB7 are analyzed for the the regulation on Rab18 using an *Arabidopsis* protoplast transient expression system.

3.6.1 The reporter driven by *Rab18* promoter is activated by ABA

The *Arabidopsis* La-er protoplasts were prepared as described in the Methods section, and the density of living protoplasts was calculated after counting protoplasts stained with FDA (Figure I-70) and adjusted to 0.5×10^6/ml. Firefly luciferase driven by the promoter of *Rab18* (Rab18-LUC) constructed in pSK vector was used as the reporter,
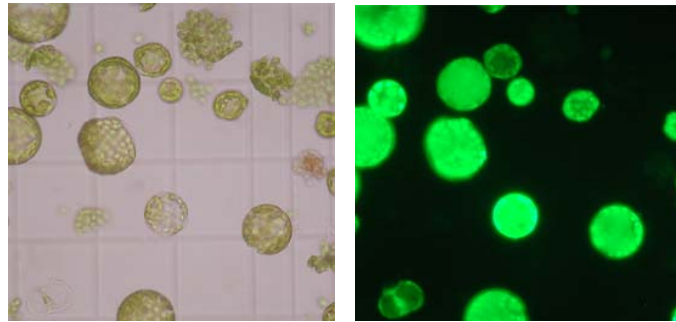
Figure I-70. Viability of *Arabidopsis* mesophyll protoplasts incubated with the live-stain FDA. The protoplasts were observed under bright field (left) and with a FITC filter to show viable fluorescent cells (right).
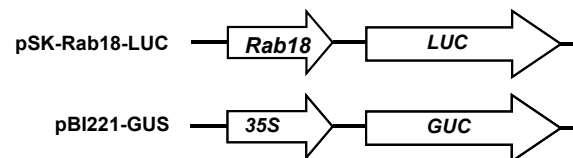
Figure I-71. Schematic presentation of the reporter constructs. The structural gene LUC and GUS are constructed as chimeric genes controlled by the promoter *Rab18* and *35S*, respectively.

and GUS driven by the cauliflower mosaic virus (CaMV) *35S* promoter constructed in

pBI221 vector was used as an internal control to normalize transfection efficiency (Hoffman, 2001; Figure I-71). 15 µg of plasmid pSK-Rab18-LUC and 10 µg of pBI221-GUS were co-transfected into 150 µl of protoplasts by PEG mediated transfection. To define the ABA-dependent expression of the reporter, 30 µM ABA was added 8hrs after transfection. LUC and GUS activity assay was performed after additional 16hrs of phenotypic expression. Analysis of LUC activity of the transfected *Arabidopsis* protoplasts revealed an approximately 10-fold induction of the expression of the LUC by 30 µM ABA (Figure I-72). It indicated that *Rab18* was a strongly ABA-induced in that experiment system.
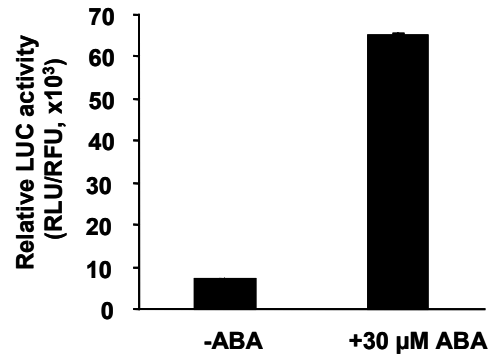
Figure I-72. The effect of ABA on the activity of LUC controlled by *Rab18* promoter in a transient gene expression system. *Arabidopsis* (La-er) protoplasts were co-transfected with 15 µg pSK-Rab18-LUC and 10 µg pBI221-GUS (as an internal control). 30 µM ABA was added 8hrs after transfection. The LUC (RLU/ sec) and GUS (RFU/ sec) activity was assayed after additional 16hrs incubation and the relative LUC activity (LUC/ GUS) is represented as the average (±SD) of three independent transfections.

3.6.2 ABI1, AtHB6 and AtGluRS negatively regulate the transcription of *Rab18*

To analyse the regulation on Rab18, the effector constructs, ABI1, AtGluRS, AtHB6 and AtHB7 structural genes controlled by 35S promoter were established in pBI221
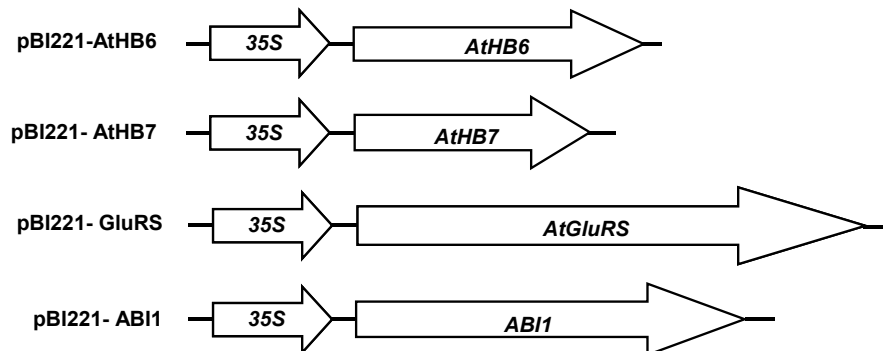
Figure I-73. Schematic presentation of the effector constructs used in transient analysis with protoplast.

pSK-Rab18-LUC, 10 µg of pBI221-GUS and different amount of effector plasmids vector (Hoffman, 2001; Yang, 2003; Figure I-73). 15 µg of reporter plasmid were co-transfected into protoplasts. The amount of total DNA in each transfection was adjusted to the same level by addition of carrier DNA.

When ABI1, AtHB6 or AtGluRS were co-transfected as effectors with the reporter Rab18-LUC, 0.2 µg of each effector DNA reduced the LUC activity slightly, and 2 µg to 20 µg of each effector plasmid DNA resulted in a striking reduction of the LUC expression (Figure I-74). Thus, ABI1, AtHB6 and AtGluRS exhibited a negative effect on the transcription of *Rab18*. These negative regulations also occurred in the presence of 30 µM ABA: 0.2 µg of each effector plasmid DNA reduced the LUC activity approximately to half of that in the control transfection. Higher amounts of ABI1 or AtHB6 resulted in more pronounced decreases in LUC activity. 4-20 µg pBI221-ABI1 completely abolished the LUC activity to background level. 20 µg pBI221-AtHB6 reduced the LUC activity to approximately 12% of the control without effector. However, increasing the amount of AtGluRS did not result in a further inhibition (Figure I-75A). These results further imply that ABI1, AtHB6 and AtGluRS are negative regulators of the *Rab18* promoter. The influence of these factors on the ABA-activated *Rab18* transcription was further characterized by the ABA-dependent induction of LUC expression. The induction of Rab18-LUC was reduced with increasing amounts of pBI221-ABI1 but was not affected by AtHB6 and AtGluRS (Figure I-75B).
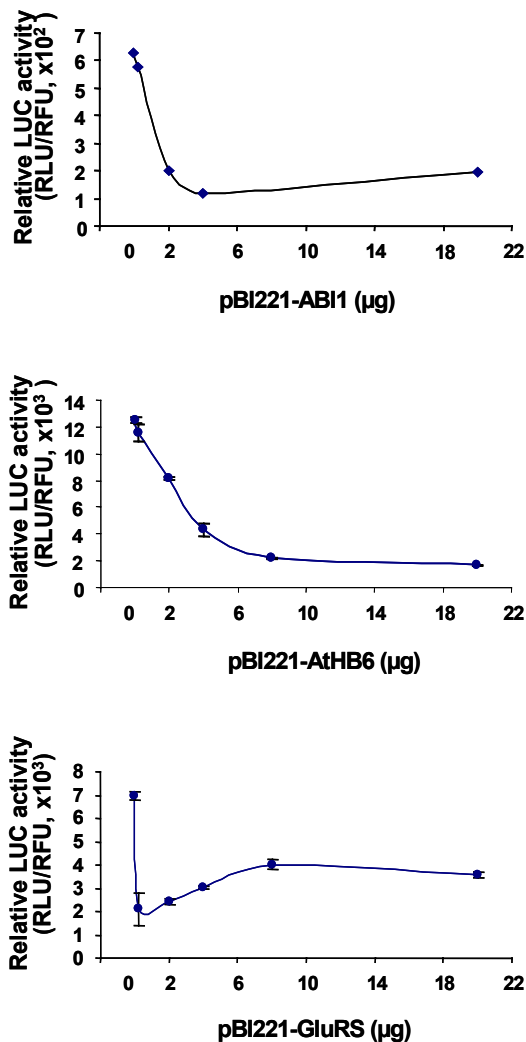
Figure I-74. The transcription of the (Rab18)-LUC is negatively regulated by ABI1, AtHB6 and AtGluRS. *Arabidopsis* (La-er) protoplasts were co-transfected with pSK-Rab18-LUC, pBI221-GUS and different amount of effector plasmid pBI221-ABI1, pBI221-AtHB6 or pBI221-GluRS. The LUC (RLU/sec) and GUS (RFU/ sec) activity was assayed after 24hrs incubation and the relative LUC activity (LUC /GUS) is given as the average (±SD) of three independent transfections. Two independent experiments yielded comparable results.

These results indicated that the transcriptional down-regulation of Rab18 expression was highly ABA-dependent with ABI1 as effector, but not ABA-dependent with AtHB6 and AtGluRS.
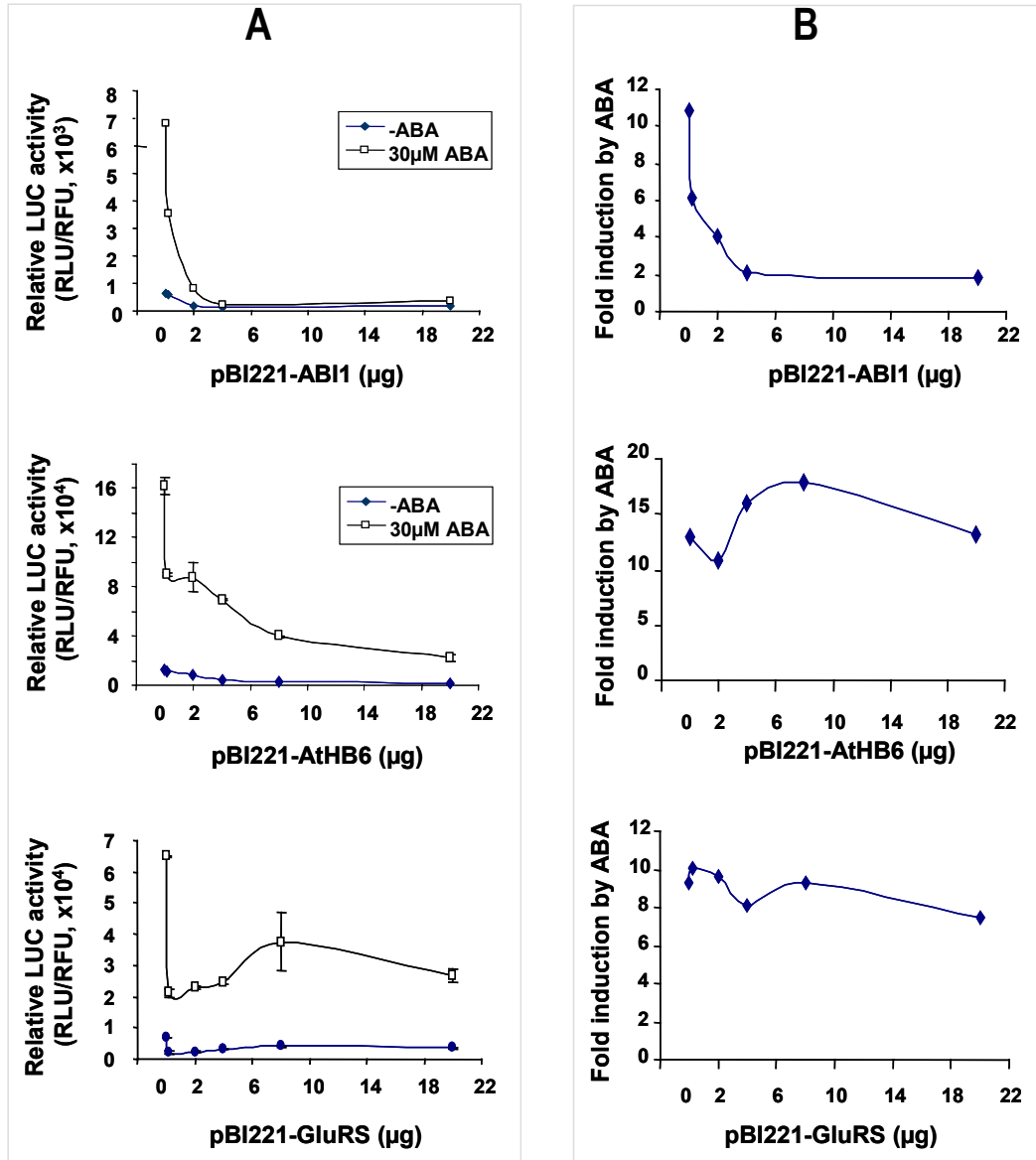
Figure I-75. The regulation of transcription of the (Rab18)-LUC by ABI1, AtHB6 and AtGluRS in the absence and presence of ABA. 150 µl *Arabidopsis* (La-er) protoplasts were co-transfected with 15 µg of pSK-Rab18-LUC, 10 µg of pBI221-GUS (for standardization) and different amount of effector plasmid pBI221-ABI1, pBI221-AtHB6 or pBI221-GluRS. 30 µM ABA was added 8hrs after transfection. The LUC (RLU/ sec) and GUS (RFU/ sec) activity assay was performed after additional 16hrs incubation. (A) The relative LUC activity (LUC/ GUS) is indicated as the average (±SD) of three independent transfections. (B) The fold induction of LUC activity by ABA is shown relative to the expression of LUC in the corresponding transfection in the absence of ABA. Two independent experiments yielded comparable results.

A

B
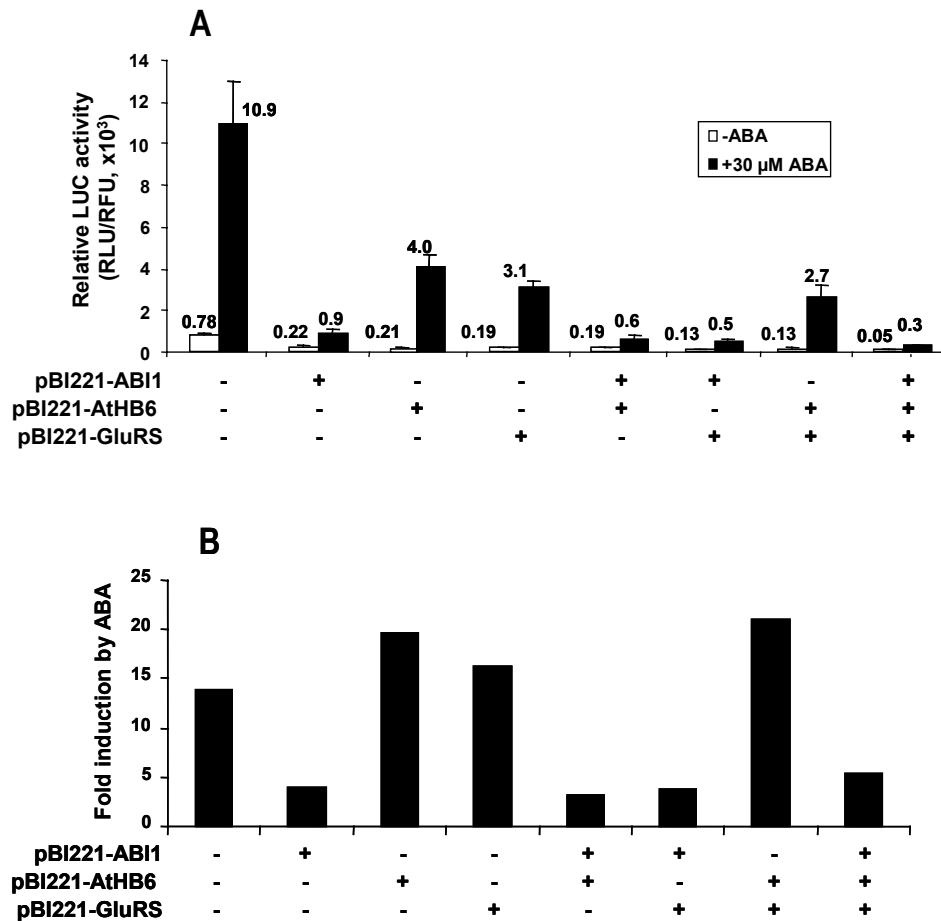
Figure I-76. The regulation of the transcription of (Rab18)-LUC by combinations of ABI1, AtHB6 and AtGluRS in the absence and presence of ABA. 150 μl *Arabidopsis* (La-er) protoplasts were co-transfected with 15 μg of pSK-Rab18-LUC, 10 μg of pBI221-GUS (for standardization) and 2 μg of each effector plasmid as indicated in X-axis. 30 μM ABA was added 8hrs after transfection. The LUC (RLU/sec) and GUS (RFU/sec) activity was assayed after additional 16hrs incubation. (A) The relative LUC activity (LUC /GUS). It was indicated as the average (±SD) of three independent transfections. (B) The fold induction of LUC activity by ABA. It is shown relative to the expression of LUC in the corresponding transfection in the absence of ABA.

Since complexes of ABI1, AtHB6 and /or AtGluRS might act in ABA signaling, combinations of these three factors were further investigated for the effect on the *Rab18* promoter. 2 μg of each effector plasmid was combined and co-transfected with Rab18-LUC. Figure I-76A shows comparable to Figure I-75 that ABI1, AtHB6 and AtGluRS each by itself apparently reduced the LUC activity. Among these three effectors, ABI1 exhibited the strongest negative effect. Combination of these effectors showed no or small additive effects. The combination of ABI1, AtHB6 and AtGluRS

resulted in even lower LUC activity as compared with each effector alone. However, AtHB6 and AtGluRS did not influence the effect of ABI1 on the ABA-induced transcription of *Rab18* (Figure I-76B).

The heterodimerization between AtHB7 and AtHB6 in yeast (Figure I-17; Figure I-18) implied that they might regulate gene expression as heterodimers. To test the function of that interaction *in vivo*, AtHB7 driven by 35S promoter was co-expressed with AtHB6 and Rab18-LUC in *Arabidopsis* protoplasts. Results showed that AtHB7 alone did not affected the transcription of Rab18-LUC (Figure I-77). It seems that AtHB7 caused partial, however, not significantly, relief of the suppression of AtHB6 on the LUC expression (Figure I-77). However, this result was not reproduced in another independent experiment.

pBI221-AtHB6	-	+	+	-
pBI221-AtHB7	-	-	+	+
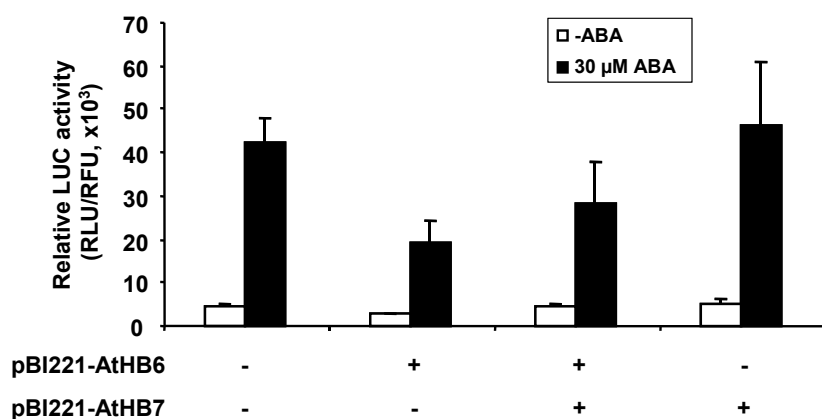
Figure I-77. The relative activity of the (*Rab1*8)-LUC affected by AtHB6 and AtHB7. 150 µl *Arabidopsis* (La-er) protoplasts were co-transfected with 15 µg of pSK-Rab18-LUC, 10 µg of pBI221-GUS (for standardization), 4 µg of pBI221-AtHB6 and/ or 10 µg of pBI221-AtHB7. 30 µM ABA was added 8hrs after transfection. The LUC (RLU/sec) and GUS (RFU/sec) activity was assayed after additional 16hrs incubation. The relative LUC activity (LUC /GUS) was indicated as the average (±SD) of three independent transfections.

3.7 Regulation of the AtHB6-activated transcription in transient expression system

Himmelbach *et al.* (2002) reported that in transgenic plants the luciferase gene controlled by the AtHB6 promoter was strongly ABA-inducible. AtHB6 could recognize a *cis*-element (CAATTATTA) present in its own promoter. The artificial promoter (designated as 4 x bs-), containing 4 copies of the AtHB6-binding sequence (CAATTATTA) fused in tandem orientation to a minimal –46 cauliflower mosaic virus

(CaMV) 35S promoter, was used to control the LUC reporter gene (Himmelbach *et al.*, 2002; Figure I-78). The expression of this reporter was increased by co-expression of AtHB6 as the effector in *Arabidopsis* protoplasts (Himmelbach *et al.*, 2002). This system was used to further demonstrate the localization and function of putative interactions between AtHB6, AtHB7, ABI1 and AtGluRS.

First, the repeated results were reproduced in both *Arabidopsis* and maize protoplasts.

Maize protoplasts were prepared as described in the Methods section and their density was adjusted to $1\text{-}2\text{x}10^6$/ml. Figure I-79 shows freshly prepared maize protoplasts. Compared with the *Arabidopsis* protoplasts, there are less collapsed protoplasts in the maize protoplast population. Maize protoplasts were transfected through electroporation.

Results confirmed that AtHB6 was able to act as a transcriptional regulator through binding on the *cis*-element present in its own promoter, and expression of the reporter is not induced by ABA (Figure I-80). It also revealed that the induction of expression of 4xbs-LUC reporter by AtHB6 was approximately 2000-fold more efficient in maize than in *Arabidopsis* protoplasts (Figure I-80).

Figure I-78. Schematic presentation of the reporter construct 4xbs-LUC in pSK. It contains four tandemly orientated copies of the AtHB6-binding sequence (CAATTATTA) fused upstream to the CaMV 35S core promoter that controls the reporter gene LUC.
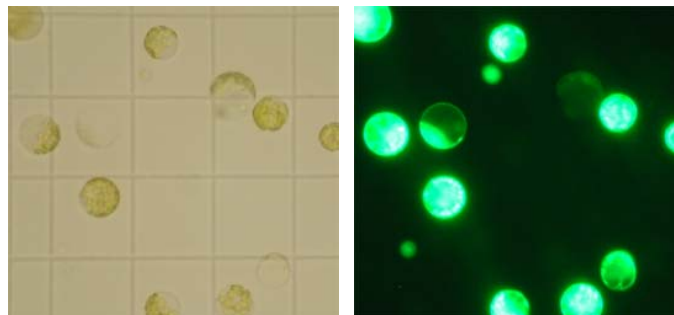
Figure I-79. Viability of maize mesophyll protoplasts incubated with the live-stain FDA. The protoplasts were observed under bright field (left) and with a FITC filter to show viable fluorescent cells (right).

A

B
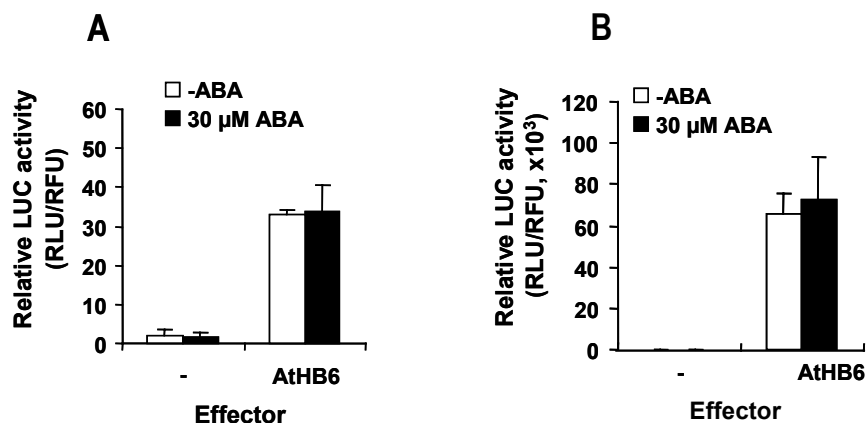
Figure I-80. The expression of (4xbs)-LUC reporter is activated by AtHB6. A: 150 µl of *Arabidopsis* (La-er) protoplasts were co-transfected by PEG with 25 µg of pSK-4xbs-LUC, 25 µg of pBI221-GUS (for standardization), and 25 µg of pBI221-AtHB6 as the effector plasmid. B: 150 µl of maize protoplasts were co-transfected by electroporation with 6 µg of pSK-4xbs-LUC, 15 µg of pBI221-GUS, and 6 µg of pBI221-AtHB6 as the effector plasmid. In both transfections, the ABA treatment (30 µM) was performed 6hrs after transfection. The LUC (RLU/sec) and GUS (RFU/sec) activity was assayed after additional 16hrs incubation. Data show that the induction of reporter expression was approximately 2000-fold more efficient in maize than in *Arabidopsis* protoplasts.

3.7.1 Effect of AtHB7 on AtHB6-activated transcription

The homeodomain protein AtHB6 could activate the expression of LUC driven by the artificial promoter 4xbs. To clarify if the interacting homeodomain protein AtHB7 is involved in AtHB6-regulated gene expression, AtHB7 effector was tested in the protoplasts transient system.

The pBI221-AtHB7 was used as the effector plasmid. The expression of LUC was not affected by co-expression of AtHB7 either in *Arabidopsis* protoplasts or maize protoplasts (Figure I-81). Surprisingly, AtHB7 does not influence the regulation of LUC expression by AtHB6 either (Figure I-81). However, this artificial promoter only contains the DNA binding sequences and does not necessarily mimic the *in vivo* situation. The full-length promoter of AtHB6 with an AtHB6 binding site might reveal a regulation by AtHB7. We used the LUC driven by the 1.4 kb promoter fragment of AtHB6 as the reporter and tried to detect the transcriptional regulation by AtHB6 and AtHB7 in protoplast. Unfortunately, no expression of LUC was detected (data not shown). The regulation of the transcription of AtHB6 might require other factors in addition to AtHB6 and AtHB7.
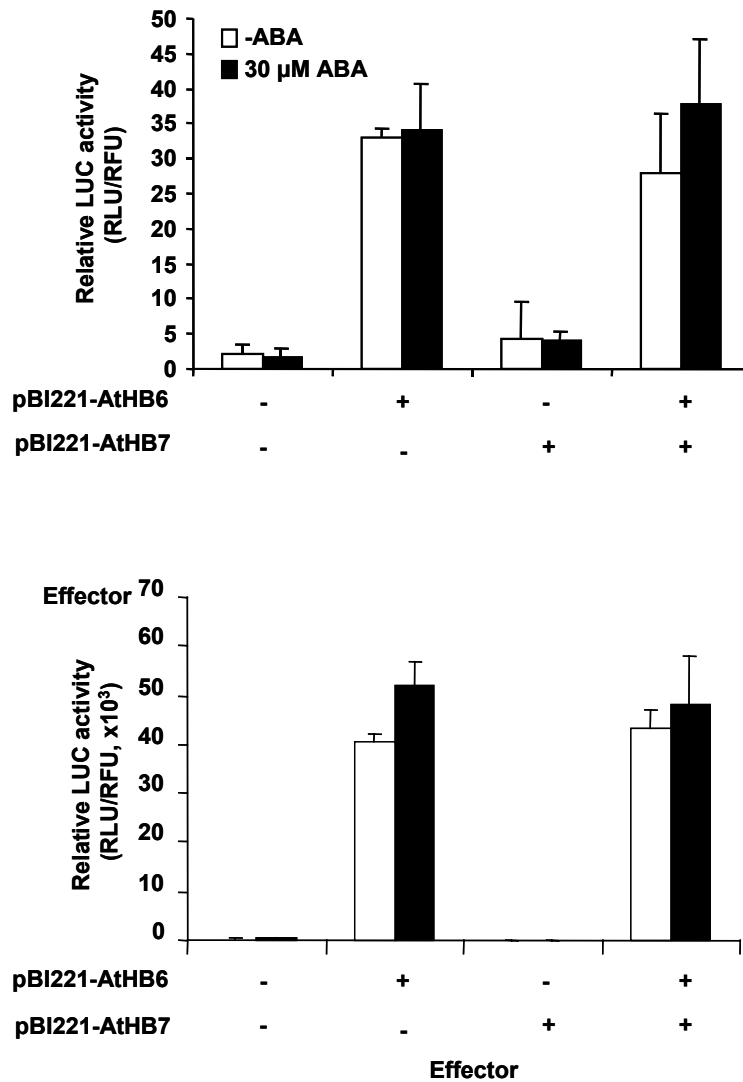
Figure I-81. The expression of LUC controlled by 4xbs was activated by AtHB6 but not by AtHB7. Upper: 150 µl *Arabidopsis* (La-er) protoplasts were co-transfected with 10 µg of each plasmids of pSK-4xbs-LUC, pBI221-GUS (for standardization), pBI221-AtHB6 or (and) pBI221-AtHB7. Bottom: 150 µl maize protoplasts were co-transfected with 4 µg of pSK-4xbs-LUC, 5 µg of pBI221-GUS, 4 µg of pBI221-AtHB6 or (and) 10 µg of pBI221-AtHB7. 30 µM ABA was added 6hrs after transfection. The LUC (RLU/sec) and GUS (RFU/sec) activity was assayed after additional 16hrs incubation and the relative LUC activity (LUC /GUS) is given as the average (±SD) of three independent transfections. Several independent experiments yielded comparable results.

3.7.2 Suppression of AtHB6-activated transcription by AtGluRS

To elucidate the effect of AtGluRS on the ability of AtHB6 to activate transcription, the 4xbs-LUC, AtHB6 and AtGluRS were co-expressed in *Arabidopsis* protoplasts as well

as in maize protoplasts.

In the *Arabidopsis* protoplasts system, 25 μg of pSK-4xbs-LUC, pBI221-GUS and pBI221-AtHB6 and (or) pBI221-GluRS were co-transformed to 150 μl of protoplasts by PEG-mediated transfection. For the ABA treatment, 30 μM ABA was added to the protoplast suspension after 6hr pre-incubation. LUC and GUS activity assay were performed after additional 16hr incubation. The results (Figure I-82) showed that the activity of the reporter LUC was approximately elevated 16-times by the co-expression of the effector AtHB6, while the co-expression of the effector AtGluRS did not affect LUC expression. Compared with AtHB6 alone, participation of AtGluRS as a co-effector showed no additional influence on the reporter expression, and ABA exhibited no effect on the expression of the reporter gene.
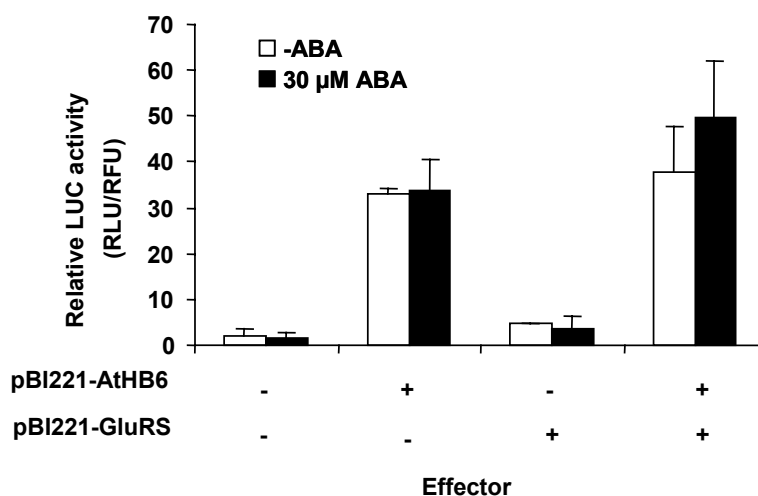
Figure I-82. The relative activity of (4xbs)-LUC affected by AtHB6 and AtGluRS. 150 μl *Arabidopsis* (La-er) protoplasts were co-transfected by PEG with 25 μg of each plasmid of pSK-4xbs-LUC, pBI221-GUS (for standardization) and pBI221-AtHB6 or (and) pBI221-GluRS as the effector. 30 μM ABA was added 6hrs after transfection. The LUC (RLU/sec) and GUS (RFU/sec) activity was assayed after additional 16hrs incubation and the relative LUC activity (LUC /GUS) was expressed as the average (±SD) of three independent transfections. Several independent experiments gave comparable results.

In further experiments, maize protoplasts were exploited to address the interactions between AtGluRS, AtHB6. For electroporation-mediated transfection, 6 μg of reporter plasmid pSK-4xbs-LUC, 15 μg of pBI221-GUS and 6 μg of pBI221-AtHB6 were transformed to 150 μl protoplasts. To survey the effect of AtGluRS on transcription of the reporter, different amounts of pBI221-GluRS were co-transfected. After transfection, 6hr incubation and additional 16hr incubation with or without ABA (30 μM) were

processed. LUC activity analysis (Figure I-83) showed that the effector AtHB6 increased the relative LUC activity (RLU/RFU) from about 170 to70000 units/second, resulting in a 400-fold induction of reporter (4xbs-LUC) expression by AtHB6. The LUC expression was reduced when AtGluRS was co-expressed with AtHB6. The higher DNA amount of pBI221-GluRS introduced, the higher the reduction of LUC expression. 10 µg pBI221-GluRS led to half reduction of LUC expression as compared with that induced by AtHB6 alone. To make sure this effect was due to expression of AtGluRS protein but not due to inhibitory influence of DNA preparation, the same amount of pBI221-GluRS DNA digested by *BamH*I (designated as D-GluRS, Figure I-84), which was unable to express a protein by splitting of the promoter 35S and cDNA AtGluRS, was co-transfected instead of pBI221-GluRS. This digested DNA showed no effect on the AtHB6-induced LUC expression (Figure I-85). As controls, AtGluRS or D-AtGluRS, was transfected in maize protoplasts as the unique effector. However, only very low LUC activity close to background level was shown and no significant change of the LUC activity was generated by the effector constructs (Figure I-86). In all these transfections, ABA showed no effect on the expression of the reporter.
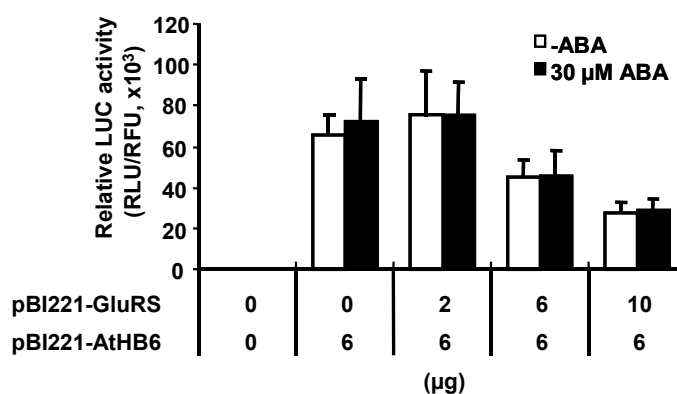
Figure I-83. AtGluRS restrains the activity of (4xbs)-LUC induced by AtHB6. 150 µl maize protoplasts were co-transfected by electroporation with 6 µg of pSK-4xbs-LUC and 15 µg of pBI221-GUS (for standardization) as the reporter, 6 µg of pBI221-AtHB6 and different amount of pBI221-GluRS as the effector. 30 µM ABA was given 6hrs after transfection. The LUC (RLU/sec) and GUS (RFU/sec) activity was assayed after additional 16hrs incubation and the relative LUC activity (LUC /GUS) is given as the average (±SD) of three independent transfections. Several independent experiments yielded comparable results.

Since the co-transfection of 10 µg of pBI221-GluRS could reduce the LUC expression induced by the 6 µg of pBI221-AtHB6 by around half. It was thought that the interaction of AtGluRS and AtHB6 restrains the function of AtHB6 possibly by

binding to AtHB6 and retaining it outside the nucleus. Thus, higher expression of AtGluRS would result in a more pronounced effect. Subsequently, pMENCHU-GluRS, in which the AtGluRS is controlled by double *35S* promoter (Figure I-87), was co-transformed with less pBI221-AtHB6 (4 μg) and less reporter plasmid pBI221-4xbs-LUC (4 μg) to the maize protoplasts. When compared with the LUC expression induced by AtHB6 alone, addition of 2 μg of pMENCHU-GluRS reduced the LUC expression by half. When the transfected amount of pMENCHU-GluRS increase to 20 μg, which was 2.1 according to the DNA molar ratio of AtGluRS/ AtHB6, the LUC expression was only reduced by no more than two-third (Figure I-88). Therefore no complete inhibition can be expected with more effector DNA of AtGluRS.
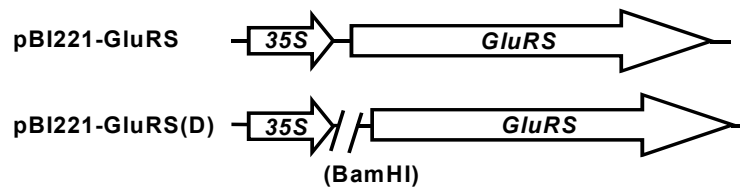
Figure I-84. The effector constructs of AtGluRS cloned in pBI221 vector and the D-AtGluRS, which is splitted of the promoter and the cDNA AtGluRS by *BamH*I.
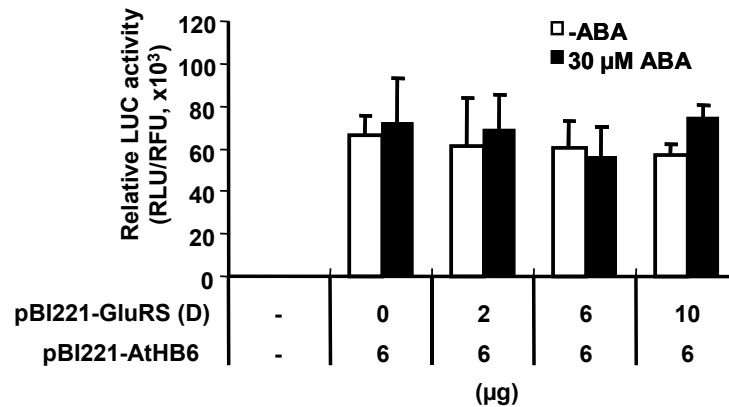
Figure I-85. The digested pBI221-GluRS which was unable to express AtGluRS could not restrains the activity of (4xbs)-LUC induced by AtHB6. 150 μl maize protoplasts were co-transfected by electroporation with 6 μg of pSK-4xbs-LUC and 15 μg of pBI221-GUS (for standardization) as the reporter, 6 μg of pBI221-AtHB6 and different amount of digested pBI221-GluRS as the effector. 30 μM ABA was applied 6hrs after transfection. The LUC (RLU/sec) and GUS (RFU/sec) activity was assayed after additional 16hrs incubation and the relative LUC activity (LUC /GUS) is given as the average (±SD) of three independent transfections.
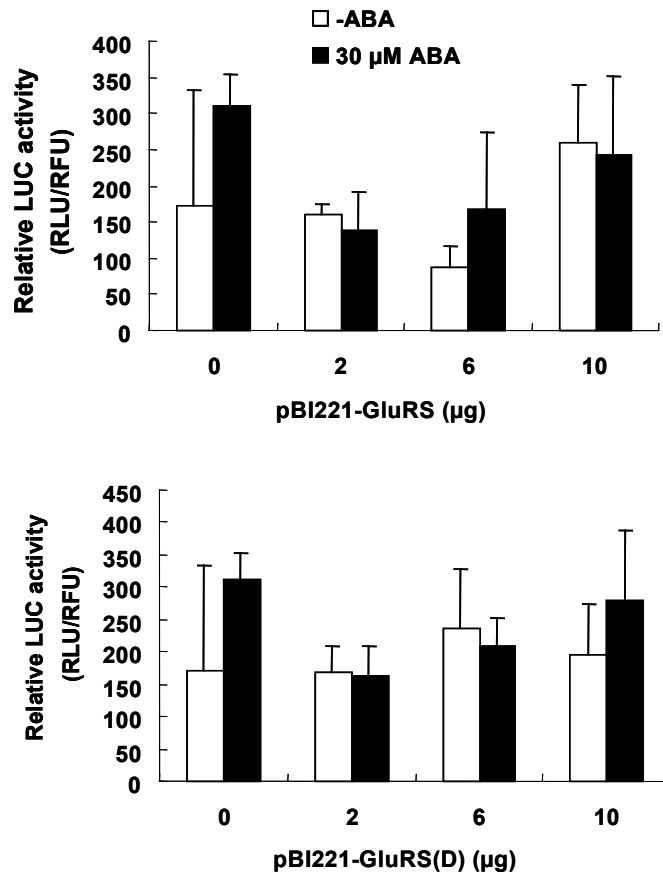
Figure I-86. Transfection with pBI221-GluRS (upper) or with the digested pBI221-GluRS (bottom) as the effector did not significantly influence the expression of LUC driven by 4xbs. 150 µl maize protoplasts were co-transfected by electroporation with 6 µg of pSK-4xbs-LUC and 15 µg of pBI221-GUS (for standardization) as the reporter, and different amount of effector plasmid DNA. 30 µM ABA was applied 6hrs after transfection. The LUC (RLU/sec) and GUS (RFU/sec) activity was assayed after additional 16hrs incubation. The value is given as the average (±SD) of three independent transfections.
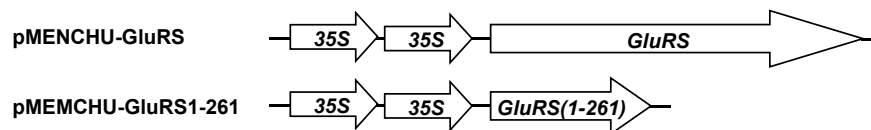
Figure I-87. Schematic presentation of the effector constructs of AtGluRS and AtGluRS(1-261) constructed into pMENCHU vector with double *35S* promoter.
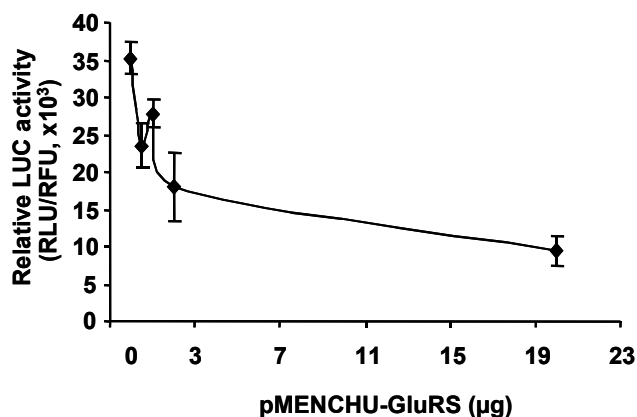
Figure I-88. AtGluRS expressed from pMENCHU-GluRS restrains the activity of LUC induced by AtHB6. 150 µl maize protoplasts were co-transfected by electroporation with 4 µg of pSK-4xbs-LUC and 5 µg of pBI221-GUS (for standardization) as the reporter, 4 µg of pBI221-AtHB6 and different amount of pMENCHU-GluRS as the effector. The LUC (RLU/sec) and GUS (RFU/sec) activity was assayed 22hrs after transfection and the relative LUC activity (LUC/GUS) was given as the average (±SD) of three independent transfections.

Provided that the inhibitory effect exerted by AtGluRS on AtHB6 trans-activation is mediated by specific protein-protein interaction, the short version of AtGluRS (GluRS1-261) should be also sufficient to play the inhibition since it was able to interact with AtHB6 in yeast two-hybrid system. Hence, GluRS1-261 was also tested of the capability of inhibiting the trans-activation of AtHB6. As the effector, 20 µg of pMENCHU-GluRS1-261 was co-transfected with 4 µg of pBI221-AtHB6 and 4 µg of pSK-4xbs-LUC in maize protoplasts. Results showed that this N-terminal version of GluRS was also able to inhibit the function of AtHB6 on gene regulation by a factor of 2. It was similar to that of the full-length AtGluRS (Figure I-89).

The results from *Arabidopsis* and maize protoplasts indicated that partial effect mediated by AtGluRS requires strong AtHB6-dependent regulation to be clearly visible. Only maize protoplast provided a suitable experiment system.

3.7.3 Stimulation of AtHB6- activated transcription by ABI1

To analyse the effect of ABI1 on the AtHB6-regulated gene expression, the effectors ABI1 and AtHB6 as well as the reporter 4xbs-LUC was co-expressed in maize protoplasts. Data showed that the activity of LUC was slightly elevated compared to that obtained with AtHB6 alone (Figure I-90). It indicates that the ABI1 likely acts as a positive regulator in the AtHB6-DNA binding event.
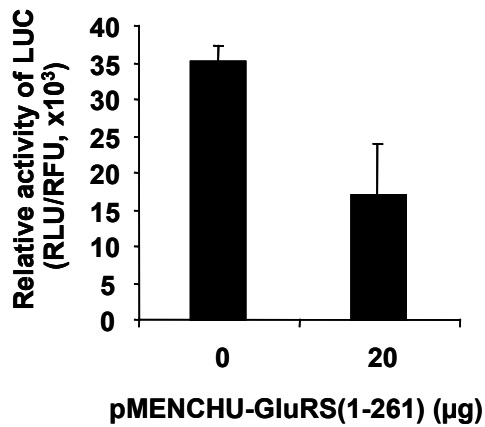
Figure I-89. Short version of AtGluRS (AtGluRS(1-261)) expressed from pMENCHU-GluRS(1-261) reduces the AtHB6-activated LUC activity. 4 µg of pSK-4xbs-LUC, 5 µg of pBI221-GUS and 4 µg of pBI221-AtHB6 were co-transfected with pMENCHU-GluRS1-261 (0 µg and 20 µg) into 150 µl maize protoplasts for expression. The LUC (RLU/sec) and GUS (RFU/sec) activity was assayed 22hrs after transfection and the relative LUC activity (LUC/GUS) was presented as the average (±SD) of three independent transfections.
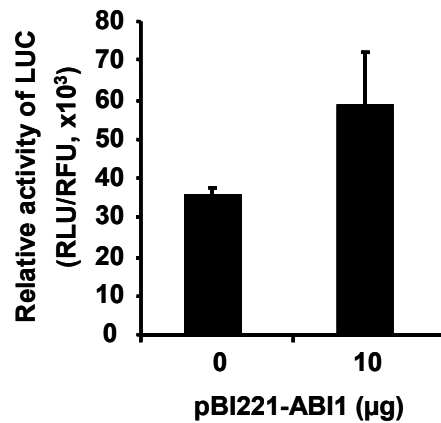
Figure I-90. The AtHB6-activated expression of LUC controlled by 4xbs was elevated by ABI1. Maize protoplasts were co-transfected with pSK-4xbs-LUC (4 µg) and pBI221-GUS (5 µg) as the reporter, pBI221-AtHB6 (4 µg) and pBI221-ABI1 (0 µg or 10 µg) as the effector. The LUC (RLU/sec) and GUS (RFU/sec) activity was assayed after 22hrs incubation and the relative LUC activity (LUC /GUS) was given as the average (±SD) of three independent transfections. Three independent experiments yielded the same tendency results.

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4   Discussion

This work presents novel data on two emerging ABA signaling elements, namely AtHB7 and AtGluRS. They were identified as interaction partners of AtHB6 by a yeast two-hybrid screen (Section 3.1). The interaction of the homeodomain transcriptional factors AtHB7 and AtHB6 (Section 3.1.3) indicated heterodimerization of the two transcriptional regulators, which might regulate response-specific gene expression. The interaction between AtHB6 and AtGluRS (Section 3.2) implies that the AtGluRS is involved in the transcriptional regulation either directly or indirectly. This would be a new function of AtGluRS besides its essential role to transfer glutamic acid onto its cognate tRNA (tRNA^{Glu}) required for protein synthesis.

The complex interaction of AtGluRS with AtHB6 and the PP2C ABI1 and ABI2 as demonstrated in the yeast system and *in vitro* (Section 3.2), the effects of AtGluRS on gene expression in protoplasts (Section 3.5.2; Section 3.5.3) and the cellular localization of AtGluRS and AtHB6 (Section 3.5.1), as well as the physiological phenotype of AtGluRS transgenic plants (Yang, 2003) support the idea that AtGluRS is deeply involved in ABA signal transduction.

In addition, the interaction of AtGluRS with itself in the yeast system supports the homodimerization of AtGluRS (Section 3.3).

4.1   Identification of protein interaction partners by yeast two-hybrid system screening

Protein-protein interactions are currently a subject of great interest. Many innovative techniques to identify interactions or complexes have been developed in recent years. As one of these techniques, the yeast two-hybrid system has provided a genetic means to identify proteins that physically interact *in vivo*. It has been used to identify novel proteins by screening expression libraries and to study interactions between known partners (Chien *et al*., 1991). These studies have been changing our perception of cellular function.

Two screens were performed in this work to identify interaction partners of AtHB6 and to find interaction partners of AtGluRS.

New interaction partners of AtHB6

In the screening for interaction partner of AtHB6, four candidates were identified from the *Arabidopsis* cDNA Ohio and the Clontech libraries using the N-terminal truncated bait AtHB6dC269 (aa 1-269). Analysis of protein sequences encoded by the identified cDNA revealed that the interacting candidates of AtHB6 include the N-terminal

sequences of AtGluRS aa –20-261 (gb accession number is AF067773, predicted 719aa) and the N-terminal part of AtHB7 aa –16-136 (emb accession number is X67032.1, predicted 256aa). In addition, nucleotides 1328-1665 of the *Arabidopsis thaliana* 18s RNA gene (emb accession X16077.1) and nucleotides 40-258 of an unknown protein gene (gene *At3g04830*, gb accession number is AF370137, predicted 299aa) were also identified as the positive candidates. The latter cDNA was, however, in a shifted frame to the GAL4 sequence, therefore a short peptide of 33 amino acids (S R G S E F A A A S T R S T N F I R F R A N R R E R D G D E N G G D T V K P T R E S S) was predicted to be encoded and this peptide has no identity with any other protein in the databank (Appendix 3).

By using the N-terminal truncated version AtHB6dC217 (aa 1-217) as the bait, only the N-terminal domain of AtGluRS was identified from the *Arabidopsis* Ohio cDNA library. No interaction candidate was found by using the very short N-terminal version AtHB6dC119 (aa1-119) truncated to present more or less the homeodomain only (Section 3.1).

The identification of AtGluRS and AtHB7 as interaction partners of AtHB6 in this system argues for the possibility that they are new ABA signaling components.

The gene family of homeodomain leucine zipper transcriptional factors have been shown to be involved in regulating plant growth and development, as well as plant responses to environmental stimuli. They encode proteins characterized by the homeodomain and the leucine-zipper domain (Ruberti *et al.*, 1991; Mattsson *et al.*, 1992; Schena and Davies, 1992).

The homeodomain leuncine zipper protein AtHB7 is deduced to have 258 amino acids (P46897; Soderman *et al*., 1994). It was characterized in the HD-ZIP class I together with AtHB1, AtHB3, AtHB5, AtHB6, AtHB12 *etc*. because of their high similarities within the homeodomains (Sessa *et al.*, 1994). AtHB7 is presented in all organs of the plant at low level (Soderman *et al*., 1996). Its expression can be induced by water deficit, osmotic stress as well as by exogenous ABA in an ABA-dependent manner. And this induction is impaired in *abi1* mutant background, indicating AtHB7 may act down stream of ABI1 in ABA signal transduction pathway which mediates a drought response (Soderman *et al*., 1996).

Abundant evidences showed that HD-ZIP proteins could form dimers. The sunflower HD-ZIP proteins, HAHB-1 and HAHB–10 were capable of homodimerization (Gonzalez *et al.*, 1997). The two HD-ZIP class II (Sessa *et al.*, 1994) proteins OsHox1 and AtHB2 from different species (Meijer *et al.*, 1997), and the two HD-ZIP class II proteins CPHB-1 and CPHB-2 from the resurrection plant *Craterostigma plantagineum*

(Frank *et al.*, 1998) form heterodimers *in vitro*. The *Arabidopsis* HD-ZIP proteins, AtHB1 and AtHB2 is able to homodimerize (Sessa *et al.*, 1993; 1997). AtHB5 was reported to form homodimers and heterodimers with AtHB6, AtHB7 and AtHB12 *in vitro* protein binding assays (Johnnesson *et al.*, 2001). In this work, the AtHB7 was identified as an interaction partner of AtHB6 *in vivo* (Figure I-17; I-18). The result suggests that they form heterodimers in yeast. Interestingly, an interaction between AtHB5 and AtHB6 as previously reported by Johnnesson *et al.* (2001) was not detected in the yeast two-hybrid assay (Figure I-18). No interaction was detected between AtHB6dC269 and different versions of AtHB6 (Figure I-15), indicating that AtHB6 does not form homodimers in yeast, or at least, the slightly C-terminal-truncated version AtHB6dC269 is not sufficient to form homodimers with itself.

Leucine zipper motifs in other classes of transcription factors are known to mediate protein dimerization (O'Shea *et al.*, 1989). The results presented here highlight that the interaction of AtHB6 and AtHB7 in yeast is highly dependent on the leucine zipper (Figure I-91). AtHB6dC269 was able to interact with AtHB7dC, which was truncated C-terminally up to the leucine zipper domain. Deletion of the C-terminal part of AtHB6 negatively affected the interaction. The C-terminal deletion up to the homeodomain completely abolished the ability for interaction with AtHB7dC (Figure I-17) as well as with AtHB7fl (Figure I-18). These results indicate that the homeodomain region in AtHB6 is not sufficient for mediating interaction with AtHB7. The leucine zipper, possibly together with its immediate C-terminal linked region, is critical for heterodimerization.

Figure I-91. Interaction strength of different versions of AtHB6 and AtHB7. +, ++ or – indicates the obvious interaction, strong interaction and no interaction, respectively. The leucine zipper is shown to be critical for the interaction.

AtHB7 / AtHB6	AtHB7dC	AtHB7fl
AtHB6dC269	+ +	+ +
AtHB6dC217	+ +	+
AtHB6dC119	-	-

HD-ZIP proteins function as transcriptional regulators by binding to specific DNA sequence through their conserved homeodomain (Gehring *et al.*, 1994) as protein dimers (Sessa *et al.*, 1993). Homodimers of AtHB1 or AtHB2 could bind to distinct 9 bp pseudopalindromic sequences (Sessa *et al.*, 1993; 1997). The formation of the dimers between AtHB7 and AtHB6, as well as between pairs of other HD-ZIP proteins could create specific combination of transcription factors for binding to specific target *cis* elements. These dimers might thus provide the potential to integrate information from different signals in the stimulus-specific regulation of gene expression.

Interaction candidates of AtGluRS

In a screen for interaction partners of AtGluRS, no positive candidate was obtained by using the complete AtGluRS protein as bait (AtGluRSfl). However, with the N-terminal part AtGluRS(1-261) as the bait, five positive candidates were isolated.

One of those positives was AtGluRS(-20-261), which also emerged in the screen with AtHB6. This indicated that AtGluRS might be able to form homodimers. GluRS from different organisms exists as the forms of monomers, dimers, or even as a component of protein complexes. For example, in *E.coli*, GluRS was found to be active as a monomer with a molecular mass of about 50 kDa (Kern and Lapointe, 1979). In wheat, three kinds of GluRS (cytoplasmic GluRS, chloroplast GluRS, and mitochondrial GluRS) were isolated as dimers and they acted as dimers that were in equilibrium with inactive monomers (Ratinaud *et al.*, 1983). In this work, the *Arabidopsis* AtGluRS, which was defined as the cytoplasmic GluRS (Figure I-65), was demonstrated to be able to homodimerize in yeast. However, when different versions of AtGluRS were tested in the yeast two-hybrid system for their abilities to interact with the baits AtGluRS(1-261) and AtGluRSfl, only the N-terminal versions AtGluRS1-216, AtGluRS(1-261) and AtGluRS1-445 displayed interaction with AtGluRS(1-261) or the full-length GluRS. The strongest interaction occurred between two AtGluRS(1-261) versions (Figure I-92). The absence of interaction in other deleted versions (AtGluRS1-110, 217-445, 232-455, 449-719) with the two tested baits implied that the region of 110-216 aa was required for the interaction, and the portion (216-261 residues) closely linked to this region plays a role in reinforcing interaction. In additional, the C-terminal part seems to interfere with the interaction in this system.

Besides AtGluRS(-20-261), 4 other interaction candidates were identified in the screen with AtGluRS(1-261) as a bait. One is the cDNA of *Arabidopsis thaliana* cytochrome p450 (dbj accession number is D78598.1, predicted 499aa). This cDNA was inserted in false-orientation to the *GAL4* activation sequences therefore encoded for a peptide of 86 amino acids with no identity to any known proteins in the data bank. The second is the

Figure I-92. The potential dimerization of different versions of AtGluRS in yeast.
Schematic diagram of the preys and baits are presented. +, ++, +/- or – indicates obvious interaction, strong interaction, very weak interaction and no interaction, respectively. The region of 110-216 might be important for homerdization.

Prey \ Bait	AtGluRS(1-261)	AtGluRSfl
AtGluRSfl	-	-
AtGluRS(1-110)	-	-
AtGluRS(1-216)	+	+/-
AtGluRS(1-261)	+ +	+
AtGluRS(1-445)	+	+/-
AtGluRS(217-445)	-	-
AtGluRS(232-455)	-	-
AtGluRS(449-719)	-	-

Arabidopsis thaliana putative cytosolic factor gene (gb accession number: AY045913.1, predicted 573aa) with a reading frame shift when compared with the open reading frame of GAL4-AD, and it coded for a peptide of 12 amino acids with no identity to known proteins. The third candidate is identical to the *Arabidopsis thaliana* rRNA repeat unit (emb: X52322.1). And the fourth is part of the At3g41970 gene which was inserted in a false direction. Noticeably, the predicted fusion proteins translated from the start code of AD of the last two candidates have high consensus to a putative senescence-associated protein ssa-13 in *Pisum sativum* (Appendix 4). The *Pisum sativum* ssa-13 (Pariasca *et al.*, 2001) cDNA was isolated from a 5-day stored pod cDNA library using differential screening. The hypothetical protein ssa-13 was found to relate to cell membrane and nutrient remobilization, to disease response-related and ribosomal proteins, and to ubiquitin-conjugating enzyme. It is likely to be involved in premature senescence of stored pea pods (Pariasca *et al.*, 2001). So far, no proteins in *Arabidopsis* have been reported to be homology with ssa-13. It is not clear if AtGluRS interacts with the possibly existing senescence-associated proteins in *Arabidopsis* and sequentially regulates the plant senescence.

Interestingly, the *Arabidopsis thaliana* rRNA repeat units was identified as the interaction candidate of AtGluRS and the 18s RNA gene was also found as a positive

interaction partner of AtHB6dC269. It is arguable how these rRNAs interact with the DBD-fusion proteins and activate the reporter genes. In principle, the reporter genes in the yeast two-hybrid system are activated only when the DNA binding domain and the activation domain is physically brought together. Thus, the explanations for the identification of rRNA as the positive candidates in this yeast system could be that the AD-rRNA fusion gene encodes an AD-fusion protein that is able to interact with DBD-fusion protein thereby activates the reporter gene, like discussed above for the rRNA repeat units. Alternatively, the *GAL4* DBD-fusion interacts with rRNA and meanwhile this rRNA binds another *GAL4* AD-containing protein of yeast. Thus the rRNA acts as a bridge and links the DBD-fusion and AD-fusion protein, as a result the reporter gene is activated (Figure I-93 A). Sengupta *et al.* (1999) and David *et al.* (2002) demonstrated the possibility of that some RNA could act as this kind of bridge, possessing the binding site for a coat protein and for another RNA-binding domain in a yeast three-hybrid system. A second alternative would be the reporter gene is activated by the RNA that interacts with the promoter-bound DBD-fusion (Figure I-93 B). Sengupta *et al.* (1999) and Saha *et al.* (2003) also proved that there were a set of RNA molecules worked as transcriptional activator when tethered to DNA through a coat protein. The presence of interaction between rRNA and AtGluRS or AtHB6 suggests that these two proteins might be involved in regulating the action of rRNA. The interaction of the cytoplasmic AtGluRS and rRNA may occur on the ribosome in cytoplasm and be involved in protein synthesis. And the interaction of nuclear localization protein AtHB6 with rRNA may implies that
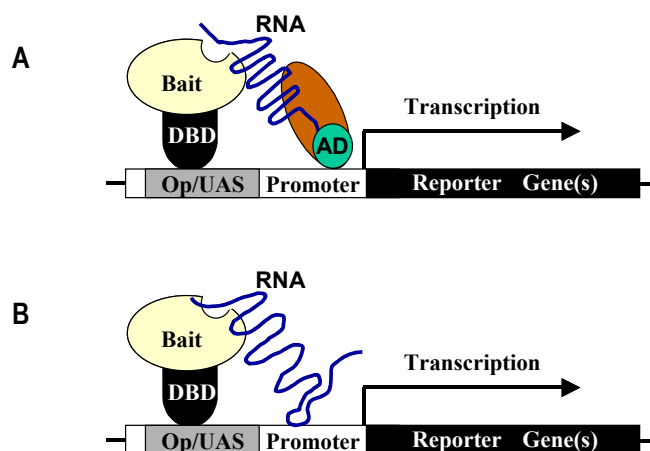
Figure I-93. Proposed model for activating the reporter by the complex of protein – RNA in yeast two-hybrid system. A: The RNA acts as a bridge and links the DBD- and AD-containing proteins, thus a transcriptional activator complex is formed. B: The DBD-fusion protein tethers the RNA that has a transcriptional activation region and forms a transcriptional activator.

the protein transcriptional factor AtHB6 is involved in the necessary steps of the rRNA processing and the ribosomal assembly in nucleus as some other protein trans-acting factors and ribosomal proteins do (Venema and Tollervey, 1999; Kressler *et al.*, 1999), or the AtHB6 can regulate special gene expression through tethering the rRNA that is able to act as the transcriptional activator (Saha *et al.*, 2003). The results argue for a novel side of ABA function in the regulating gene expression.

Most interactions detected in the yeast two-hybrid system are testimony for the usefulness of the experimental tool. However, not all the *bona fide* proteins interactions can be detected in this system. For example, despite the highly cooperative nature of the interactions between yeast cytochrome bc_1 components (Hunte *et al.*, 2000), or the interaction of the human electron transfer flavoproteins (ETF) α and β subunits observed between soluble proteins (Roberts *et al.*, 1996), two-hybrid interactions were not detected. In this study, the N-terminal region of AtGluRS(1-261) is able to interact with ABI1, ABI2, AtHB6 and AtHB7. However, the full-length AtGluRS displayed no detectable interaction with AtHB6 (Figure I-21), AtHB7 (Figure I-24), and ABI1 (Figure I-28). A similar result was also reported for a partial-length AtGluRS that was screened as a positive interaction partner of a cyclin protein *cyc1bAt*, while no further interaction was detected for the full-length AtGluRS (Day *et al.*, 1996). It seems that the C-terminal part of AtGluRS diminished the interaction ability of its N-terminal region in yeast. However, the *in vitro* interaction (Figure I-35) as well as protein co-immunoprecipitation (Figure I-44, Figure I-45) demonstrated the definite interaction occurring between AtGluRS and ABI1/ *abi1*. Likewise, the *in vivo* function of AtGluRS (Section 3.6) also showed the close relationship between the full-length AtGluRS and AtHB6. Thus, the absence of detectable interaction in the yeast system could reflect improper folding of the full-length AtGluRS in yeast, or steric hindrance of full-length AtGluRS protein to be brought into close physical proximity the promoter region, or the full-length AtGluRS interacts with those tested protein but the complex cannot be imported into the nucleus to activate the reporter because of the large molecular mass of the complex. However, the full-length AtGluRS could not reduce the expression of the reporter gene activated by DBD-AtHB7. The result suggested that the AtGluRS of this version could not interact with AtHB7. A complementation assay for function of the AtGluRS in yeast failed and indicated that the AtGluRS cannot substitute for the yeast GluRS. All these findings imply that the improper folding or steric hindrance of full-length AtGluRS protein are most probably the reasons for the undetectable interaction in yeast.

These results emphasize the limitations of the yeast two-hybrid system, even though the significant contribution it has provided to protein interaction studies (Chien *et al.*, 1991; Von Mering *et al.*, 2002).

4.2   Interaction of AtGluRS with AtHB6 and PP2C

The homeodomain of AtHB6 is sufficient for the interaction with AtGluRS

Either HD-ZIP proteins or AtGluRS are able to form dimers or complexes with other proteins (Johnnesson *et al.*, 2001; Himmelbach *et al.*, 2002; Ratinaud *et al.*, 1983; Cotelle *et al.*, 2000; Section 3.3). In this study, AtGluRS was identified as an interaction partner of AtHB6 in a yeast two-hybrid screen, suggesting complex formation between these two proteins. Further studies showed that the N-terminal short version of AtHB6 truncated up to the homeodomain was still sufficient to interact with AtGluRS, while the C-terminal of AtHB6 might act to support the interaction. Similar results were obtained from analyses of the interaction between AtHB7 and AtGluRS (Figure I-23). Thus the homeodomains in these HD-ZIP proteins are critical for the interaction with AtGluRS. Only the AtGluRS(1-261) was able to interact with AtHB6, and all other versions (AtGluRS1-110, 1-216, 217-445, 232-455, 449-719), even the full-length AtGluRS, displayed no detectable interactions with AtHB6 in yeast. Hence, the N-terminal region (1-261 residues) in AtGluRS seems to be responsible for the interaction. The proper folding of AtGluRS might be an essential factor for the interaction as discussed in section 4.1.

It is the first time that a relation between *Arabidopsis* GluRS and HD-ZIP proteins is described. The major role of the homeodomain in HD-ZIP proteins was believed to be binding to specific DNA sequence in order to mediate transcriptional regulation (Gehring *et al.*, 1994). The interactions between homeodomains of AtHB6/ AtHB7 and AtGluRS illustrates a novel function in protein-protein interaction and suggest that the AtGluRS might be involved in regulation of gene transcription.

Interaction of ABI1 with AtGluRS not depending on its phosphatase domain

Yeast two-hybrid assay revealed that the N-terminally deleted ABI1dN as well as the reduced phosphatase activity versions abi1dN and ABI1NAP had strong two-hybrid interactions with AtGluRS(-20-261) (Figure I-27). Comparable interactions were also detected *in vitro* between AtGluRS and ABI1/ abi1 (Figure I-35). These data suggest that the catalytic activity of ABI1 not discriminates with respect to the interaction with AtGluRS. The N-terminal deletion of ABI1 (ABI1dN262) showed apparently reduced, but still strong interaction with AtGluRS, while C-terminal truncation of ABI1 (ABI Sac) still containing the protein phosphatase 2C signature (aa 172 – 180) completely abolished the interaction (Figure I-27). This implies that the C-terminal region (262-434 amino acids) of ABI1 is a major determinant for the interaction. The interaction of ABI1

and AtGluRS was further demonstrated by the *in vivo* co-immunoprecipitation assay based on the co-expression of interacting proteins tagged by different epitopes in maize protoplasts, confirming the *bona fide* interaction of ABI1 and AtGluRS.

The interaction of AtGluRS and ABI2 (Yang, 2003) was also confirmed in this study (Figure I-29). However, no interaction was detected between AtGluRSdC and the mutant type abi2. It indicates that the functional catalytic domain in ABI2 probably plays an important role in the interaction with AtGluRS.

It was reported that some of the aminoacyl-tRNA synthetase activities present in high molecular mass complexes might be regulated by phosphorylation/dephosphorylation events (Berg, 1990). The GluRS protein complexed with other aminoacyl-tRNA synthetases specific for arginine, aspartic acid, glutamine, isoleucine, leucine, lysine, methionine, and proline isolated from rabbit reticulocytes could be phosphorylated by protein kinase C (Venema and Traugh, 1991). Although the catalytic activity of ABI1 is not critical for the interaction with AtGluRS, the dephosphorylation of AtGluRS by ABI1 cannot be excluded since the dephosphorylation by ABI1 probably occurs after the interaction event. The importance of the catalytic domain in ABI2 for the interaction with AtGluRS also implied the possibility of dephosphorylation. Moreover, the N-terminal part of *Arabidopsis* GluRS shows some sequence similarity to translation elongation factor EF-1 gamma (Day *et al.*, 1998). The phosphorylation of EF-1 gamma by MPF (maturation promoting factor), a universal regulator of the G2 to M transition of the cell cycle, has been reported by Janssen *et al.* (1991). The similar sequence in AtGluRS may suggest that AtGluRS can be phosphorylated/dephosphorylated. Furthermore, analysis of the *Arabidopsis* GluRS by ScanProsite program (Falquet *et al.*, 2002) defined 29 serine/threonine phosphorylation sites which have the potential of dephosphorylation by the PP2Cs.

A recent study showed that the enzymatic activity of ABI1 was significantly reduced in the presence of AtGluRS when the concentration of Mn^{2+} was below 2 mM (Yang, 2003). Thus interactions of AtGluRS with ABI1 or ABI2 might also inactivate the enzymatic activity of ABI1 or ABI2 by altering their affinity to Mn^{2+} or Mg^{2+} (Yang, 2003).

It should be mentioned that much stronger interactions occurred between AtGluRS(-20-261) than AtGluRS(1-261) with AtHB6 or ABI1 (Figure I-20; I-21; I-27; I-28). The additional 20 amino acids (SLSQTHIAQSISRFSDLDPK) appended to the N-terminal seem to strengthen the interactions apparently. When standard protein-protein BLAST (Altschul *et al.*, 1997) was used to analyse this peptide sequence, no significant similarity was found. Further 'searching for short nearly exact matches' of

this peptide revealed a short fragment in a transcriptional regulator in LuxR family (*Vibrio parahaemolyticus*) with 75% identity:

Query: 9   QSISRFSDLDPK   20

QSI   RFS+ L+PK

Sbjct: 156   QSIFRFSNLNPK 167

This short peptide may enhance the transcription of reporter genes in the yeast two-hybrid system.

The phosphatase catalytic domain of ABI1 is critical for the interaction with AtHB6

Himmelbach *et al.* (2002) reported that the interaction of AtHB6 with ABI1 in the yeast two-hybrid system and *in vitro* was dependent on the phosphatase activity domain in ABI1. This result was further confirmed in this work: Strong interaction occurred between AtHB6 and the C-terminal domain of ABI1, reduced interaction was found between AtHB6 and the C-terminal domain of *abi1*, and no interaction was detected between AtHB6 and the non-active protein phosphatase NAP (Figure I-27). Furthermore, no interaction was detected between AtHB6 and the C-terminal truncated ABI1 (ABI1 Sac, positions1-268aa), indicating that both the functional catalytic domain and the C-terminal region (268-434aa) of ABI1 are required for the interaction with AtHB6. The weak interaction of AtHB6dC269 with ABI2 and no interaction of AtHB6dC269 with *abi2* also indicate that the catalytic domain in ABI2 is required for the interaction. No interaction was detected between AtHB7 and ABI1 (Himmelbach *et al.*, 2002), and between AtHB5 or AtHB7 and ABI2 (Figure I-26), supporting the idea that the interaction between AtHB6 and ABI1 or ABI2 is specific.

The C-terminally deleted version AtHB6dC269 displayed a severely reduced ability to interact with ABI1 (Figure I-25). This indicates that not only the homeodomain region (Himmelbach *et al.*, 2002) but also the C-terminal part (269-311 residues) of AtHB6 is critical for the interaction. There is one potential casein kinase II phosphorylation site (SGEE, 285-288) in this C-terminal part (ScanProsite, 2003). It is not clear if this phosphorylation site is critical for the interaction with ABI1. To clarify it, point-mutation and *in vivo* and *in vitro* phosphorylation assay should be helpful.

The transcription of AtHB6 has been reported to be activated by ABA in an ABI1-dependent manner (Soderman *et al.*, 1999). In animals, the phosphorylation status regulates protein association, DNA-binding ability or subcellular compartmentation (Whitmarsh and Davies, 2000). The phosphorylation of AtHB6 by PKA *in vitro* abolished specific binding to the *cis*-element (Himmelbach *et al.*, 2002). ABI1 enhanced the

AtHB6-activated gene transcription in the transient expression experiment (Figure I-90) demonstrating biologically that the presence of ABI1 or the interaction of ABI1 and AtHB6 is helpful for the function of AtHB6. And the de-phosphorylation of the AtHB6 by co-expression of PP2C ABI1 is believed to promote binding of AtHB6 with DNA and enhance the AtHB6-activated gene transcription.

Ternary interactions detected in a yeast three-hybrid system

In higher eukaryotic organisms GluRS is capable to associate in large multiprotein complexes. In yeast, GluRS and methionine tRNA synthetase (MetRS) interact with Arc1p which functions as a scaffold protein of these synthetases *in vivo* (Simos *et al.*, 1996; Galani *et al.*, 2001). Until now, multienzyme complexes containing GluRS have been obtained from sheep, rabbit, rat, bovine, drosophila and yeast (Freist *et al.*, 1997; Ting *et al.*, 1992; Galani *et al.*, 2001). The complex formation might enhance their catalytic efficiency (Deinert *et al.*, 2001; Galani *et al.*, 2001). Furthermore, signaling complexes or "transducisomes" were reported from yeast and animal to provide a structural basis for specificity in signaling (Pawson and Scott, 1997). In *Arabidopsis*, it was demonstrated that AtGluRS binds to scaffolding protein 14-3-3s (Cotelle *et al.*, 2000). In plant, 14-3-3 members located within the nucleus are constituents of transcription factor complexes and interact with components of ABA-induced gene expression machinery (Fulgosi *et al.*, 2002). The presented results suggest that AtGluRS has the ability to interact with protein phosphatases ABI1 and ABI2, and also interacts with homeodomain proteins AtHB6 and AtHB7. These findings highly support for a role of AtGluRS in ABA signal transduction.

The yeast three-hybrid system allows investigation of ternary protein interactions, such as ternary complex formation, prevention or reinforcement of the interaction between two previously defined proteins by inhibitors or by modifiers (Tirode *et al.*, 1997). It can also be used to discover indirect interactions mediated by a third protein. Apparent interferences of AtHB7, ABI1, abi1, ABI2 or abi2 with the interaction between AtHB6dC269 and AtGluRS(1-261) were detected (Section 3.2.4). It is obvious that the competence of AtHB7 to AtHB6 in interacting with AtGluRS is based on their high similarity in structure. The interferences of ABI1, abi1, ABI2 and abi2 should be carefully considered. Since AtHB6dC269 interacts very weakly with ABI1 and abi1, while AtGluRS strongly interacts with ABI1 and abi1, it is supposed that AtHB6 and ABI1/ abi1 compete in interacting with AtGluRS and therefore weaken the interactions with AtGluRS by each other. Likewise, the presence of AtGluRS would weaken the interaction of AtHB6 and ABI1 (Figure I-94). ABI1 (Gosti *et al.*, 1999; Merlot *et al.*, 2001; Sheen, 1998; Tahtiharju and Palva, 2001) and AtHB6 (Himmelbach *et al.*, 2002) were found to act as negative regulators in ABA signaling, while the over-expression of

AtGluRS in *Arabidopsis* made the plants more sensitive to ABA (Yang, 2003,). The AtGluRS might function as antagonist of AtHB6 or/ and ABI1 through interacting with these proteins and disturbing the interaction between AtHB6 and ABI1. Nonetheless, this postulation is not suitable for ABI2, since even though no interaction between abi2 and AtHB6 or AtGluRS was detected the interference of abi2 with the interaction of AtHB6 and AtGluRS was observed (Figure I-50). Moreover, a role of the phosphorylation/ dephosphorylation event cannot be ruled out, because the expression of the wild type ABI1 or ABI2 as the third protein reduced the interaction of AtHB6-AtGluRS more severely than the abi1 or abi2 did according to the expression of *LacZ* reporter. The yeast growth inhibited by the expression of the third protein abi1 or abi2 could be rescued by the application of histidine while it was not the case for the third protein ABI1 or ABI2.
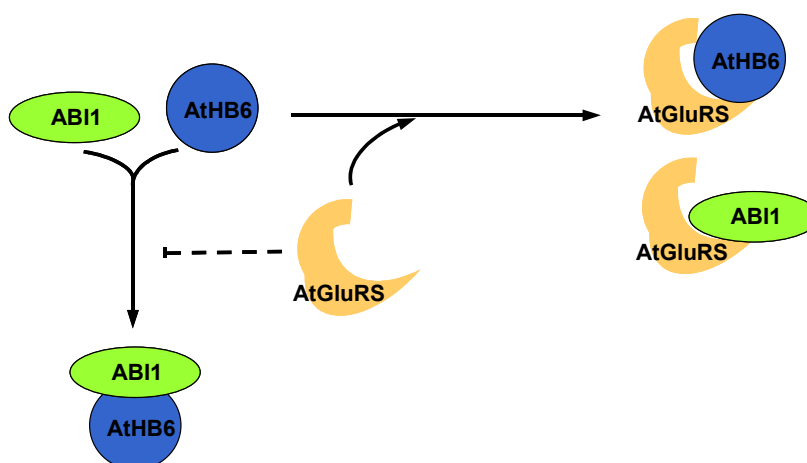
Figure I-94. Ternary interaction among ABI1, AtHB6 and AtGluRS. Interactions can occur between every two of them. ABI1 and AtHB6 compete to interact with GluRS, therefore the presence of AtGluRS disturbs the interaction between ABI1 and AtHB6.

4.3   Function of AtGluRS

AtGluRS and its essential functions

GluRS has been cloned and sequenced from many organism including bacteria (Russell and Pittard, 1971; Breton *et. al.*, 1986), yeast (Tzagoloff and Shtanko, 1995), animals (Cerini *et al.*, 1991) and plants (Ratinaud *et al.*, 1983; Day *et al.*, 1998). It belongs to the class I aminoacyl-tRNA synthetases, which commonly has the conserved consensus tetrapeptide His-Ile-Gly-His ('HIGH') at the putative ATP binding site for adenylating (Brick *et al.*, 1989) and Lys-Met-Ser-Lys-Ser ('KMSKS') at the putative binding site for the 3' end of the tRNA. (Freist *et al.*, 1997).

In plants, several GluRS were isolated from chloroplasts, mitochondria and the cytoplasm of wheat (Ratinaud *et al.*, 1983; Thomes *et al.*, 1983), and chloroplast of barley (Andersen, 1992) and tobacco (accession number X83524). In *Arabidopsis thaliana*, the AtGluRS (2.3 kb) gene was screened from an *Arabidopsis* cDNA library. It was found to own 6 exons, encodes for a protein of 719 amino acids with a mass of 81 kDa. It's more likely the cytoplasmic GluRS according to the size (Day *et al.*, 1998). The AtGluRS screened in the yeast two-hybrid system is identical to this gene (Section 3.1.2; Section 3.3.2). Two conserved motifs in Class I aaRSs present in this AtGluRS are the 'HIGH' motif (residues 227-230) and the 'KMSKS' motif, which has been changed to 'LSKRK' (residues 449-453) (Day *et al.*, 1998).

Comparing the *Arabidopsis* AtGluRS with GluRS in yeast, human, and bacteria, the closest homology occurs with the yeast GluRS. The homology is highest from the 'HIGH' motif up to the C-terminal end of the protein (Figure I-57). Surprisingly, when the *Arabidopsis* GluRS was tested for complementation of GluRS-deficient yeast strain, no rescue was obtained (Figure I-61, I-62).

It is well known that the GluRS plays an essential role in protein synthesis. It covalently links the glutamic acid to its cognate tRNA ($tRNA^{Glu}$):

$$GluRS + Glu + ATP \xrightleftharpoons{\quad tRNA^{Glu} \quad} GluRS\text{-}Glu\text{-}AMP + PPi$$

$$GluRS\text{-}Glu\text{-}AMP + tRNA^{Glu} \rightleftharpoons GluRS + Glu\text{-}tRNA^{Glu} + AMP$$
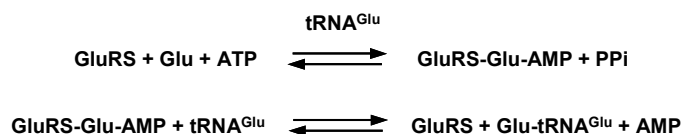
In the chloroplasts of plants, green algae, cyanobacteria, and in some eubacteria and archaebacteria, the C5-pathway is operated to synthesize δ-ALA, which is the precursor of porphyrin (Ilag *et al.*, 1994). The plastidic GluRS plays a crucial role in this pathway:

Glutamate + $tRNA^{Glu}$

⬇ *glutamyl-tRNA synthetase*

Glu-$tRNA^{Glu}$

⬇ *glutamyl-tRNA reductase*

glutamate-1-semialdehyde

⬇ *glutamate-1-semialdehyde aminomutase*

δ -ALA

Except for its essential role in protein synthesis, little is known about other postulated functions of GluRS in cellular. For example, other aaRSs have been reported to have specific roles in: (1) transcriptional regulation. (2) translational regulation. (3) mitochondrial RNA splicing. (4) tRNA maturation proofreading/export. (5) cytokine-like activity (review, Martinis *et al.*, 1999). However, until now, no report
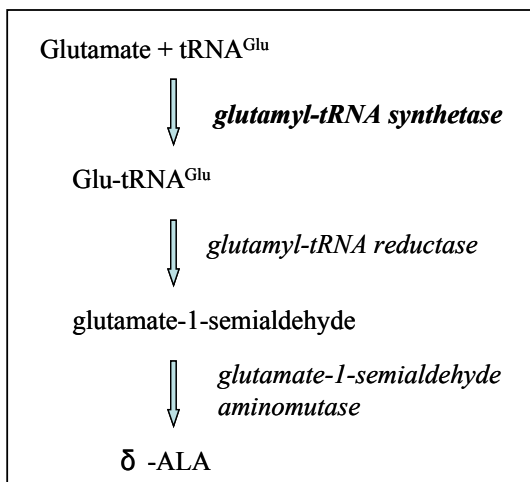
has yet shown that GluRS exhibits one of these roles. Galani *et al.* (2001) reported that when the yeast GluRS was inhibited to assembly into a complex with MetRS and Arc1p, significant amounts of MetRS, GluRS and Arc1p could enter the nucleus. This may suggests that the GluRS has some functions in the nucleus even though no functional proof has been provided so far.

AtGluRS regulates the function of AtHB6 by retaining it in the cytoplasm

The interactions between HD-ZIP proteins and AtGluRS suggest that the AtGluRS might be involved, at least indirectly, in regulation of gene transcription. The protoplast transient expression assay has been frequently used to study protein-protein interaction (Subramaniam *et al.*, 2001) and stress and hormone signaling (Sheen, 1996, 1998; Kovtun *et al.*, 1998, 2000; Hwang and Sheen, 2001; Himmelbach *et al.*, 2002). This system was used for addressing the possible regulation of AtHB6-regulated gene transcription. Analysis of the transient expression of LUC controlled by 4 copies of the AtHB6-binding sequence (4xbs-) in maize protoplast showed that the LUC expression induced by AtHB6 was suppressed at the most of 50-70% by co-expression of either the full-length version of AtGluRS (Figure I-83, I-88) or the truncated version of AtGluRS(1-261) (Figure I-89). AtGluRS interacting with AtHB6 and forming a complex protein was suspected to be the reason for the negative regulation. More AtGluRS might be able to capture more AtHB6 and thus suppress more AtHB6-regulation. However, AtGluRS expressed from pMENCHU-GluRS driven by a double 35S promoter, transfected at 2.1 times of AtHB6 according to the DNA molar, was unable to suppress the AtHB6-induced expression totally (Figure I-88). The reason for this might be that the expression of AtGluRS is controlled and maintained at a certain level and no further overexpression occured. As the transcription of LUC driven by the AtHB6 promoter was demonstrated to be ABA- and ABI1-dependent in transgenic plants and the transgenic *Arabidopsis* with over-expression of AtHB6 is insensitive to ABA (Himmelbach *et al.*, 2002), AtHB6 is deduced to be a negative regulator of ABA signaling. The presented results suggest that the AtGluRS is involved in ABA signaling as a positive regulator by modulating the function of AtHB6. Since the AtGluRS did not influence the expression of the reporter when it was expressed as the effector alone (Figure I-85), it is unlikely that AtGluRS acts directly on the promoter of AtHB6 and regulates the transcription of AtHB6.

In *Arabidopsis* protoplasts, although the over-expression of AtHB6 could induce the expression of 4xbs-LUC (Figure I-80), the additional expression of AtGluRS showed no effect on the reporter expression (Figure I-82). These results are definitely different from those obtained in maize protoplast. It seems that partial effect mediated by AtGluRS requires strong AtHB6-dependent regulation. Or the expression or regulation of these

genes displays different behavior in the protoplasts from homologous species and distant species. In *Arabidopsis* protoplast, homologous proteins of AtHB6, like the HD-ZIP proteins AtHB1, AtHB5, AtHB7 *etc*. (Sessa *et al.*, 1994), might compete in the interaction with AtGluRS (Figure I-23) therefore weaken the effect of AtGluRS on AtHB6. These results reveal the complex signal network present in the organism.

  Proteins that are imported into the nucleus often contain a classical nuclear localization signal (NLS) consisting primarily of a pattern of basic residues. AtHB6 has a bipartite nuclear targeting sequence 'RRDNESLLQEISKLKTK' (Dingwall and Laskey, 1991) at residues of 141-157 and it was reported to be localized in the nucleus (Himmelbach *et al.*, 2002). ABI1 also has a monopartite nuclear motif (KPRRK) targeted at its C-termini (423-427 residues) and has been localized in nucleus (Himmelbach, personal communication). Does the AtGluRS, the interaction partner of these two proteins, also localize in the nucleus? The potential classical NLSs searching (Figure I-95) revealed that the baker yeast (*S. cerevisiae*) cytoplasmic GluRS has the predicted NLSs 'KHKK' (555-558) and 'PKHKKNP' (554-560), fission yeast (*S.pombe*) cytoplasmic GluRS has 'PRHKK', and *Arabidopsis* GluRS has 'KHKK' (550-553) and 'PKHKK' (549-553), while no putative NLSs are found in the corresponding bacteria GluRS. Galani *et al.* (2001) reported that yeast GluRS could be localized in the nucleus when it was prevented from forming a complex with MetRS and Arc1p. If the complex formed, it was distributed in the cytoplasm. This may suggests that the GluRS has some functions in the nucleus as well as in cytoplasm. However, up to day no evidence supports that the *Arabidopsis* GluRS is nuclear localized. On the contrary, AtGluRS fused to GUS was observed specks distribution in the cytoplasm and exclusively out of the nucleus in the analysis of the intracellular localization (Figure I-65).

Figure I-95. The putative nuclear localization signals (NLSs) in GluRSs. The NLSs are marked by red square.

The effect caused by over-expression of AtGluRS on the cellular distribution of AtHB6 was assayed by monitoring the localization of the AtHB6-GUS fusion protein through the histological analysis for GUS activity. Similar to AtHB6-GUS transgenic plant cells (Himmelbach *et al.*, 2002), GUS activity in F1 seedlings from crosses of

AtHB6-GUS line to wild type (RLD) was exclusively detectable in the nucleus, while in root cortex cells and guard cells of F1 seedlings from crosses of the AtHB6-GUS line to the AtGluRS-overexpression lines, however, the AtHB6-GUS fusion protein displayed localization in both cytoplasm and nucleus (Secton 3.5). This revealed that the cellular compartment of AtHB6-GUS was altered from nucleus to both the cytoplasm and nucleus by the over-expression of AtGluRS. There are two possible reasons for explaining these results. One possibility is that the over-expression of AtGluRS induced the expression of AtHB6 and the highly abundant AtHB6 was not completely imported into the nucleus in time hence accumulated also in the cytoplasm. Alternatively, part of the AtHB6-GUS was retained in the cytoplasm by the over-expression of cytoplasmic AtGluRS. In the first case, the over-expression of AtGluRS would accelerate the transcription or expression of AtHB6 and stimulate the function of AtHB6. However, the AtGluRS transgenic plants did not show the distinguished elevation of the transcription of AtHB6 (Yang, 2003). In addition, the transcription regulation of AtHB6 was not stimulated but was suppressed by the co-expression of AtGluRS in a maize protoplast transient expression system (Figure I-83; I-88; I-89). Thus the second explanation seems to be more tenable. It is an interesting hypothesis that the cytoplasmic AtGluRS interacts with AtHB6 or ABI1 and captures and retains them in the cytoplasm. The retaining of AtHB6 in the cytoplasm could reduce the import of AtHB6 into the nucleus thus diminish the transcriptional regulation of AtHB6. Accordingly, the negative regulation of AtHB6 in ABA signaling is reduced. For that reason, plants with over-expression of AtGluRS exhibit an ABA-hypersensitive phenotype that was indeed demonstrated by Yang (2003).

4.4   A model for the functions of AtGluRS, AtHB6 and ABI1 in ABA signaling

ABA can triger the transcription or /and activation of much ABA signal components that comprises the negative and positive ABA regulators. The positive regulators, *e.g.* some protein kinases (Li *et al.*, 2000; Gomez-Gadenas *et al.*, 1999; Burnett *et al.*, 2000), and the negative regulators, such as the G proteins and the protein phosphatases (Himmelbach *et al.*, 2003), interfere with each other negatively and function co-ordinately to regulate a series of physiological events and finally induce the ABA-responses (Figure I-96A).

There are plenty of findings evidenced that the ABI1 and AtHB6 are negative regulators in the ABA signaling. In the protoplast transient expression analysis the down-regulation of ABI1 and AtHB6 on the ABA-inducible Rab18-LUC reporter expression was also proved (Figure I-75, I-76). In this study, AtGluRS was demonstrated to be able to interact with AtHB6 as well as ABI1 (Section 3.2) and suppress the trans-activation of AtHB6 (Section 3.6). To elucidate the interactions
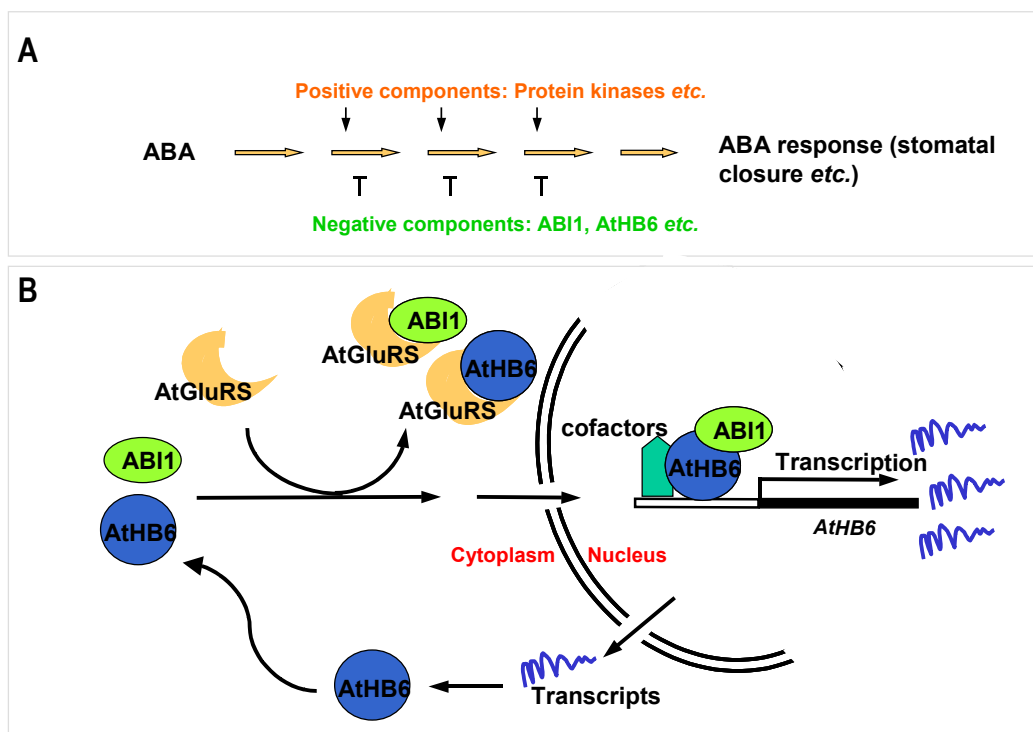
Figure I-96. Simplified model for proposed functions of AtGluRS, ABI1 and AtHB6 in ABA signalling. A: The ABA signal transduction components includes negative and positive ABA regulators, they interfere with each other negatively. B: ABI1 and AtHB6 are negative regulators in ABA response. As an interaction partner of AtHB6 and ABI1, AtGluRS captures AtHB6 and/or ABI1 in the cytoplasm and results in a blocked signal relay from ABI1 to AtHB6 and inhibition of the trans-factor function of AtHB6. The negative regulation of ABI1 and AtHB6 on ABA response is further suppressed. Thus, AtGluRS displays a positive effect on the ABA signaling.

among AtGluRS, AtHB6 and ABI1 and the functions of these proteins in ABA signaling, a model for the ABA signal transduction pathway is proposed (Figure I-96B). As illustrated in the figure, the ABI1 enhances the trans-factor function of AtHB6, whatever through de-phosphorylation event or acting as the co-trans-factor. The interaction of AtGluRS and AtHB6/ ABI1 probably occurs in the cytoplasm. The complex formation retains the AtHB6, maybe also the ABI1, in cytoplasm and reduces the opportunity of nucleus import of AtHB6, as a result, the trans-factor function of AtHB6 is decreased. A new function of AtGluRS is unraveled: it prevents AtHB6 from functioning as a transfactor therefore acts as a positive regulator in the ABA signal pathway. However, the present evidences are not sufficient enough for comprehending the relationship between ABI1 and AtGluRS. It is also unclear whether the AtHB6 activates or represses the transcription of its own. Much transcriptional factor *e.g.* AtHB7 and so on might be involve in this transcriptional regulation together with AtHB6. Further study on the function and localization of ABI1 affected by AtGluRS, the ABA responses of plants

with overexpression of AtHB6/ ABI1 affected by AtGluRS, as well as analysis of the regulation of gene transcription controlled by full-length promoter of AtHB6, will be helpful to clarify these problems.

4.5 Does AtHB7 regulate the gene transcription as the AtHB6?

AtHB6 and AtHB7 are similar in various aspects. Both AtHB6 and AtHB7 belong to HD-ZIP class I (Sessa *et al.*, 1994). Their expression was markedly induced by water deficiency and abscisic acid (Söderman *et al.*, 1996; Söderman *et al.*, 1999; Himmelbach *et al.*, 2002). Furthermore, the induction of both genes was seriously impaired in the ABA-insensitive mutant *abi1*, and the induction of AtHB6 was also reduced in *abi2* background (Söderman *et al.*, 1996; Söderman *et al.*, 1999; Himmelbach *et al.*, 2002; Hoth *et al.*, 2002). These findings imply that expression of AtHB6 and AtHB7 are dependent on the *ABI1* and (or) *ABI2* genes. Both of them possibly act downstream of ABI1 and (or) ABI2 and are involved in the ABA signal transduction.

The two-hybrid interaction assay in yeast demonstrated that AtHB7 and AtHB6 could form heterodimers (Figure I-17, I-18). It was supposed that the heterodimerization between AtHB7 and AtHB6 could thus integrate information and increase the level of complexity in transcriptional regulation at the protein level in ABA signaling pathways. Previous study has already demonstrated that AtHB6 was able to recognize a *cis*-element (CAATTATTA) present in its own promoter and subsequently activated the gene transcription under the control of these AtHB6-binding sequences (4xbs) in an ABA-dependent manner (Himmelbach *et al.*, 2002). Whereas the co-expression of AtHB7 in *Arabidopsis* or maize protoplast neither interfered with the AtHB6-activated reporter (4xbs-LUC)'s transcription nor influenced the reporter's transcription directly (Figure I-81). However, the promoter used in this assay is rather artificial. Studies of the expression of LUC under the control of the *Rab18* promoter revealed that the AtHB6 negatively regulated the ABA-induced gene expression, while the AtHB7 did not show similar regulation (Figure I-77). Hence, the function of the heterodimerization of AtHB6 and AtHB7 remains unclear.

4.6 Is ABI1 conjugated with a small protein in plant

The expected molecular weight of ABI1 is 48 kDa and of cMyc-ABI1 is 49 kDa whereas western blots analysis of proteins expressed in maize protoplast demonstrated that either the cMyc-ABI1 or the ABI1 displayed an apparent molecular mass of around 60 kDa. Results suggest that additional protein (s) of molecular mass of >10 kDa is conjugated with ABI1. What is this small protein is unclear. A recent paper (Lois *et al.*, 2003) reported that small ubiquitin-like modifiers (SUMO) of molecular mass of ~12

kDa in *Arabidopsis* modulates the ABA signal transduction pathway, these small proteins were confirmed to be conjugated to high molecular mass proteins and form conjugated with a molecular mass of >60 kDa. ABI1 is possible covalently conjugate with small proteins like SUMO. If this is true, a new regulatory aspect of ABI1 protein stability emerges.

Part II

Suppressor screen with an auxin-resistant mutant *axr1-12* of *Arabidopsis thaliana*

1   Introduction

1.1   Fertilization and parthenocarpy

In most angiosperms, fertilization requires pollen grains germination on and pollen tubes penetration through the stigma. Growth of the pollen tube towards the ovule is taking place in the stylar tissue and finally reach the embryo sac for double fertilization. In the embryo sac one of the sperm cells unites with the egg cell to form the zygote, and the second sperm cell unites with the diploid central cell to produce the triploid endosperm. In the majority of flowering plants, fruit set is dependent on successful completion of pollination and fertilization (Gillaspy *et al.*, 1993). Following fertilization the ovule develops into a seed while the surrounding carpel and in some species, other floral organs like sepal, petal or stamen differentiate into fruit tissue (Coombe, 1976; Pratt, 1988). If fertilization does not occur, the entire flower senesces. Following the abscission of other floral organs, the carpel development ceases and no fruits set (Granell *et al.*, 1992; O'Neill and Nadeau, 1997).

  However, in some species, under special conditions, the fruit can develop in the absence of fertilization, this is called parthenocarpy. Parthenocarpic fruits are devoid of embryo and endosperm. Parthenocarpy can resulted from: 1) ovary development without pollination, like in citrus and banana; 2) fruit growth stimulated by pollination but without fertilization, like in certain orchids; 3) fertilization followed by abortion of the embryos, like in grapes, peaches and cherries (Frank and Cleon, 1985). In nature, such fruits do not occur frequently, because they do not yield viable offspring and thus do not contribute directly to fitness. Of the respective species parthenocarpy can be a result of a breeding programme or may be artificially induced by exogenous application of hormones. Several edible fruits such as grapes and watermelon, have been bred as seedless fruit, and some evidence suggest that the increased auxin and gibberillin levels in ovary contribute to parthenocarpy (Ozga *et al.*, 2002; Rotino *et al.*, 1997; Acciarri *et al.*, 2002; Williamson *et al.*, 1995; Iwohori *et al.*, 1968; Mapelli *et al.*, 1978; Mazzucato *et al.*, 1998; Fos *et al.*, 2000).

1.2   Fruit growth is dependent on phytohormones synthesized in the developing seed

Ovule's fertilization and seed's development are usually considered to be essential determinants of fruit growth (Nitsch, 1950; Archbold and Dennis, 1985) because the fertilized ovules (or developing seeds) synthesize high levels of plant growth hormones, stimulate cell division, and lead to fruit set and growth (Eeuwens and Schwabe, 1975; Mapelli *et al.*, 1978; Ben-Cheikh *et al.*, 1997; Rodrigo *et al.*, 1997).

Auxins

Much evidence suggests that auxin is a major determinant of fruit development.

   Auxin is produced in pollen, in the endosperm and in the embryo of developing seeds, and the initial stimulus for fruit growth may result from pollination. Successful pollination initiates ovule growth, which is known as fruit set. After fertilization, fruit growth seems to depend on auxin produced in developing seeds. The endosperm probably contributes the auxin signal during the first stage of fruit growth, and the developing embryo may take over the fraction as the main auxin source during the latter stage (Taiz and Zeiger, 1998). Ozga *et al.* (2002) reported that seed removal resulted in rapid decreases in pericarp growth of young pea (Pisum sativum), while auxin (4-chloroindole-3-acetic acid) or GA application could maintain the growth in deseeded pericarp similarly to pericarps with seeds by obtaining the same sizes of mesocarp cells. Some other reports also showed that the fruit growth can be sustained by application of auxin to substitute the developing ovules (Nitsch, 1950; Schewabe and Mills, 1981). This evidence supports well that auxin is responsible, at least partially, for fruit development initiated by fertilization. In stimulating the formation of parthenocarpic fruits, auxin may act primarily to induce fruit set. Consequently, an endogenous increment in auxin synthesis and content within the ovules during early stages of floral and fruit development might be a way to support fruit setting and growth without pollination. This was evidenced by parthenocarpic fruit development in transgenic tobacco and eggplants expressing *DefH9-iaaM* gene, which codes for tryptophan monoxygenase and is able to increase auxin biosynthesis in transgenic plant cells and organs (Rotino *et al.*, 1997; Acciarri *et al.*, 2002).

Gibberellins

In some cases, applications of gibberellins can cause fruit set and growth of some fruits, where auxin may have no effect. Fruit growth of tomato could be induced by the application of GAs to unpollinated ovaries (Schwabe and Mills, 1981; Sawhney, 1984). Fruit containing GA-producing seeds are larger than those with GA-deficient seeds (Groot *et al.*, 1987). Parthenocarpic fruit set and development is induced by exogenous application of gibberellins in blueberry (*Vaccinium* spp.) (Williamson *et al.*, 1995), grape (*Vitris vinifera* L.) (Iwohori *et al.*, 1968) and peach (*Prunus persica* L.) (Stembridge and Gambrell, 1972). In tomato, natural parthenocarpic fruits contain more GA-like substances than their non-parthenocarpic counter-parts early after anthesis (Mapelli *et al.*, 1978), one recessive parthenocarpy mutant *pat* can synthesize 160 times higher levels of GA_{20} than normal (Mazzucato *et al.*, 1998; Fos *et al.*, 2000). Elevated levels of endogenous GAs have also been observed in the fruits of mandarins plants exhibiting naturally occurring parthenocarpy (Talon *et al.*, 1990; 1992), and tomato

fruits induced by the application of 4-chlorophenoxyacetic acid (Koshioka *et al.*, 1994).

However, other plant hormones are also involved in fruit growth. For instance, ethylene is known to influence fruit development, and some of the effects of auxin on fruiting may be mediated through the promotion of ethylene synthesis (Taiz and Zeiger, 1998).

The above evidence indicates that the fruit development can be uncoupled from fertilization and seed development. The elevation of phytohormones in fruit tissue other than seeds itself may be sufficient to induce fruit development. We also presume that alteration of the sensitivity to some plant growth regulators might be one way to obtain fruit development without fertilization. Furthermore, the cross-talking presents between different plant hormones. For example, exogenous Auxin (IAA) could restore the endogenous level of GA_1 which was reduced in the stem stump of pea by decapitation (Ross *et al.*, 2000). Application of high concentration of auxin enhanced ethylene biosynthesis in etiolated *Arabidopsis* (Woeste *et al.*, 1999) and an *Arabidopsis* mutant *alh1* displays defects in both ethylene and auxin response (Vandenbussche *et al.*, 2003). And Auxin can affect the localization of the ABA-dependent gene expression in *Arabidopsis* (Suzuki *et al.*, 2001), while ABA inhibits IAA-induced cell elongation (Pilet, 1989). These findings suggested that each plant development process might involve different plant hormones signalling. However, little is known about the plant hormone signal transduction and the molecular events in the initiation of fruit development. Hence, related mutant screening is one of the best ways to identify the genes involved in controlling fruit development.

1.3 *Arabidopsis*: an attractive model plant in biology

Since the 1980's, *Arabidopsis* has been widely used as an attractive laboratory organism for studying molecular genetics in plants. This plant is in small size, and has a short life cycle and is easily grown and bred. Especially, it owns remarkably small genome which contains a complete set of genes, that is about 26,000 genes, for controlling developmental patterns, metabolism, responses to environmental cues and disease resistance. It has a low content of repetitive-sequence DNA (Leutwiler *et al.*, 1984; Meyerowitz, 1987). These traits greatly facilitate gene identification, cloning, and sequencing.

Seed and fruit development in *Arabidopsis*

In *Arabidopsis*, the megaspore mother cell undergoes meiotic divisions to form four megaspores. Three of these degenerate, and one undergoes mitotic divisions to form the cells of the embryo sac. The embryo sac consists of seven cells, which contain eight

nuclei: the egg, two synergids, a single diploid central cell and three antipodals. When fertilization occurs, one sperm cell fuses with the egg cell to form the zygote, and a second sperm cell fuses with the diploid central cell to produce the triploid primary endosperm nucleus. Following double fertilization, the polarized zygote comes through a series of stages: quadrant, globular, heart, torpedo and upturned-U maturation (Taiz and Zeiger, 1998; Bowman, 1994). During these stages, an axis of polarity is fixed, shoot and root meristems are formed, the storage organs are generated. In contrast, the primary endosperm nucleus mitotically divides and produces a syncytium of nuclei. These nuclei migrate within an expanding central cell, after which cytokinesis occurs, and endosperm nuclei and cytoplasm are partitioned into cells. The endosperm may not only produce storage proteins, starch, and lipids for nourishing the developing embryo (Bowman, 1993, Kiyosue et al, 1999), but also play other roles including hormonal regulation of embryo, maintenance of a high osmotic potential around the embryo etc. (Bowman, 1993).

The fruits of *Arabidopsis* are siliques consisting of two congenitally fused carpels separated by a false septum. After fertilization, the cells in defined layers of the carpel divide, expand and differentiate to form the exocarp, mesocarp, structural sclerenchyma and endocarp of the silique (Vivian-Smith and Koltunow, 1999). Longitudinal growth of the silique occurs by cell expansion in all layers, however, mesocarp formation is also characterized by cell division. Siliques elongate rapidly after pollination to support the development of the rapidly growing seeds, reaching their maximum length two or three days after fertilization. The siliques become yellow from green about one week following fertilization, and then brown at maturity.

Silique development does not commence if pollination does not occur. However, this is not always the case. For example, mature *embryo-defective 24* plants with 100% fully aborted seeds can be obtained *via* tissue culture and develop normal fruits (Franzmann *et al.* 1989). Parthenocarpic silique development can also occur in *Arabidopsis* following the application of exogenous phytohormones (Jacobsen and Olszewski, 1993; Chaudhury *et al.*, 1994). Vivian-Smith and Koltunow (1999) found that naphthylacetic acid (NAA) could cause development of the unfertilized pistil by modifying the exocarp and mesocarp cell expansion, and gibberellic acid (GA_3) induce the silique development by promoting cell elongation in the exocarp and cell division in the mesocarp and endocarp.

The mutant isolation in *Arabidopsis* has been proven useful for identifying the genes controlling fruit and seed development and studying the molecular events governing fruit and seed development.

A mutant named *fruitfull*(*ful-1*) with a loss-of-function mutation in the MADS-box

AGL8 gene was isolated by Gu *et al.* (1998). This mutation blocks the elongation of siliques after fertilization and results in crowded siliques full of seeds. This showed that the MADS-box gene is required for the normal pattern of cell division, expansion and differentiation during morphogenesis of the *Arabidopsis* silique.

5 mutants with fertilization-independent seed or endosperm development have also been isolated too. 3 genes were identified coding for proteins repressing seed development in the absence of pollination and fertilization. They are *FIS1* (Luo *et al.* 1999) which is identical to *MEDEA* (Grossniklaus *et al.* 1998; Kiyosue *et al.* 1999), *FIS2* (Chaudhury *et al.* 1997; Luo *et al.* 1999) and *FIE* (Ohad *et al.* 1996; 1999; Chaudhury *et al.* 1997). *MEA* encodes a SET domain type POLYCOMB protein (Grossniklaus *et al.* 1998), FIS2 protein has a putative C2H2 zinc-finger motif, it probably encodes a transcription regulator. FIE is 40% identical to Polycomb group genes that encode a WD domain, such as extra sex combs (*Esc*) gene from Drosophila which controls pattern formation during embryogenesis (Gutjahr *et al.* 1995). Ohad *et al.* (1999) proposed that in *Arabidopsis*, the FIE protein may interact with other Polycomb proteins (like MEA) to form complexes and repress the transcription of genes required for replication of the central cell nucleus and subsequent endosperm pre-fertilization development.

Auxin-resistant mutant *axr1-12* in *Arabidopsis*

Arabidopsis can be used to identify the genes controlling hormone signal transduction (Hobbie, 1998; Phillips, 1998). In *Arabidopsis*, five auxin response loci (*AXR1*, *AXR2*, *AXR3*, *AXR4*, *AUX1*) have been identified by screening mutants that are resistant to auxin (Maher and Martindale, 1980; Lincoln *et al.*, 1990; Wilson *et al.*, 1990; Hobbie and Estelle, 1995; Leyser *et al.*, 1996; Nagpa *et al.*, 2000). These genes have been characterized at the molecular level.

The AXR1 protein is required for rapid auxin responses (Timpte *et al.*, 1995; Abel *et al.*, 1995). It encodes a protein related to the ubiquitin-activating enzyme E1, so it is likely to have a role in protein degradation (Leyser *et al.*, 1993).

axr1-12 is an auxin-resistant mutant which in addition to the auxin resistance, displays reduced hypocotyl elongation in the dark, lack of apical hook formation, a reduction in apical dominance, and defects in leaf, inflorescence, and flower morphology. It produces significantly less pollen, and fails to elongate the filaments, and as a result, it exhibits greatly reduced fertility and does not produce siliques normally (Estelle and Somerville, 1987; Lincoln *et al.*, 1990). Hence, it can be used to screen for mutant in silique development. Since *axr1-12* is affected in auxin response, mutants that stimulate the auxin signaling independent of *AXR1* could be suitable candidates.

1.4 Suppressor screen

Suppressor isolation is one of the best ways to further investigate gene function and to define additional loci (or genes) that affect the same developmental process as the original mutation. This requires generating a large seed supply of the original mutant to be mutagenized, generating an M1 (the first progeny of mutated plants) and M2 (the second progeny of mutated plants), and screening for a modified or restored mutant phenotype. Suppressor analysis has been used in a number of systems. In *Arabidopsis*, suppressor screens have been used to identify genes involved in auxin response (Cernac *et al.*, 1997), ABA biosynthesis (Koornneef *et al.* 1982) and gibberellin signal transduction (Jacobson and Olszewski, 1993; Wilson and Somerville, 1995). In a screen for suppressors of the ABA-resistant seed germination phenotype of the *Arabidopsis abi1-1* mutant, Beaudoin *et al.* (2000) recovered the ethylene-mutant *ein2*. Suppressor screens may thus also help to detect interactions between signaling cascades of different hormones.

The main purpose of this work is, by using *axr1-12* as the original mutant, to isolate suppressors which are hyper-sensitive to auxin or GA and are able to develop seeds and/ or siliques without fertilization (parthenocarpy), and to find additional loci that control these development processes predicted to be highly dependent on hormone signaling.

2   Materials and Methods

2.1   Plant materials and growth conditions

axr1-12, the original mutant, is an auxin insensitive mutant, derived from Columbia ecotype (Estelle and Somerville, 1987; Lincoln *et al.*, 1990).

Plants were grown in a growth chamber with a 14-hr day length, a light intensity of 120 µmol s^{-1}m^{-2}, at 22 $^{\circ}$C, 65% relative humidity (day) / 18 $^{\circ}$C, 75% relative humidity (night). Plants were sown in 10- x 10- x 10-cm pots, filled with nutrient soil and supplied with tap water every 2 to 4 days.

2.2   Screen

Growth regulator solution

2,4-D, GA$_3$, BAP was dissolved in minimium volume of 2 M NaOH, ethanol, and 2 M HCl, respectively. The dissolved hormones were then buffered in 0.1 mM MES. ACC (1-aminocyclopropane-1-carboxylic acid) was dissolved directly in 0.1 mM MES. The pH was adjusted to 6.5. 0.04% (v/v) Triton X-100 was added as a surfactant.

Emasculation

Flowers were emasculated approximately 1 to 2 days pre-anthesis. To avoid damage to the inflorescence meristem from emasculation, extra-fine scissors were used to remove sepals, petals, and anthers, leaving an exposed pistil covered by a thin polyethylene film.

Pollination

Pollination was performed 1 to 2 days after emasculation by dusting a freshly dehisced anther over the extended stigmatic papillae until pollen was seen adhering to the stigmatic surface.

Application of plant growth regulators (PGRs)

For screening, the flowering *axr1-12* plants (at 31-32 days after sowing) were sprayed with PGRs once daily for 2 consecutive days. 5 to 7 days after the treatment, the silique development was evaluated.

For pistil treatment, 1 µl of the respective PGR solution was applied 2 days after the emasculation to each pistil. Controls were made by the corresponding solutions without growth regulators.

Determination of the pistil growth

Final silique length was measured at 5 days after treatment. Growth data for each treatment were collected by the examination of 5 to 8 individual pistils.

Screen

0.75 g, estimated 40,000 *axr1-12* seeds, were soaked for 16 hrs in 100 ml 0.3% (v/v) EMS (methanesulfonic acid ethyl ester), and then washed in water over a period of 4 hrs. These M1 seeds were sown at a density of approximately 1 seed/ cm^2 on nutrient soil. At maturity, the plants that could get normal siliques were harvested separately as the putative mutants. The other plants were bulk harvested to produce the M2 seed pools. Seeds from about 1000 M1 plants were collected as one M2 pool. When screening in M2, flowering M2 plants were sprayed once daily for 2 consecutive days with 2,4-D or GA_3. 5 days later, the putative mutants that could develop siliques were selected. The putative mutants were again tested in the next generation for auxin response of root and silique development to confirm the phenotype.

2.3 Determination of auxin sensitivity

The surface sterilized seeds (80% EtOH/ 0.1% Triton X-100 for 30 minutes, then 3.5% hypochlorite/ 0.1% Triton X-100 for 1 minute, and washed 5 times in sterilized water.) were distributed on the surface of MS agar (10 g/l) medium plates. Plates were placed in a culture room in a vertical position so that the roots could grow along the agar surface. After 4 days, seedlings were transferred to new plates supplemented with various concentrations of 2,4-D and allowed to grow for additional three days more. The root elongation was measured and the percent root growth inhibition was calculated relative to the root elongation on the MS medium without hormone. At least 15 seedlings per treatment were analyzed.

2.4 Water loss

Four-week-old well-watered plants were used for measuring the speed of water loss from detached leaves. 3 rosette leaves on 5 different plants were excised at time after start of light perceived in growth chamber. Detached leaves were then weighed under ambient conditions every 5 minutes and water loss was calculated relative to the initial weight. 5 individual plants of each line were measured as repeats.

2.5 Germination ratio

Approximately 100 seeds were surface sterilized and distributed on the surface of MS agar plates. The germinated seeds were counted 5 days later.

2.6 Apical hook and root gravitropism

Surface sterilized seeds were aligned on the MS medium plates. Plates were covered with aluminum film to create dark conditions and incubated in the culture room in a vertical position. Apical hook formation and root gravitropism were observed 4 days later.

2.7 DNA micro-isolation from *Arabidopsis* leaves:

DNA was isolated from *Arabidopsis* leaves according to Rogers and Bendich (1994). Briefly, 3-5 young *Arabidopsis* rosette leaves (around 1 g fresh weight) were collected, homogenized in 100 µl micro isolation buffer, and mixed vigorously with additional 500 µl micro isolation buffer. The mixture was incubated at 65 ^{o}C for 60 min. 750 µl CHCl$_3$ was then added and mixed vigorously with the buffer solution. After centrifugation (1,4000 rpm, 20 min) the upper phase containing DNA was transferred to a new eppendorf tube and the DNA was precipitated by 750 µl isopropanol. The pellet was then dried and incubated with 50 µl 1xTE containing 20 ng/µl RNAse at 37 ^{o}C for 1hr to digest the RNA. The DNA was again precipitated by EtOH and dissolved in 20 µl 0.1xTE for PCR test.

Solutions:

Extraction buffer:
0.1 M	Tris-HCl(pH 8.0)
5 mM	EDTA (pH 8.0)
0.35 M	Sorbitol

Nucleus lysis buffer:
200 mM	Tris-HCl (pH 8.0)
50 mM	EDTA (pH 8.0)
2 M	NaCl
2%	CTAB (w/v)

100 ml   Micro isolation buffer (pH 7.5):
41.67 ml	extraction buffer
41.67 ml	nucleus lysis buffer
16.67 ml	5% sarcosyl
0.38 g	sodiumdisulfite

2.8 PCR

Two primers were designed for amplifying the genomic fragment covering *axr1-12* mutation. The PCR was performed using 2 µl DNA extracts of the leaves as the template, and GTGCACTTGCCTCTATGG and CAATCACAACATCTTATGGC as the forward and reverse primers, respectively.

2.9 Genetic analysis

The genetic analysis was done to determine the linkage between the new mutation and the *AXR1* gene. Mutants were crossed to the original mutant *axr1-12* or to wild type Col, and the resulted F1 plants were allowed to self-pollination to generate an F2 population. To determine the segregation, the auxin-response of root growth and the phenotype of adult plants were assayed in the F2 population.

To test if mutants are allelic, crosses between mutants were performed and the auxin-response of roots was assayed in the F1 generation.

3   Results

3.1 Silique growth of the original *axr1-12* mutant and the wild type plants in response to PGRs

Silique development of *Arabidopsis* has different response to PGRs as demonstrated by Vivian-Smith and Koltunow (1999). To analyze the silique growth of the auxin insensitive mutant *axr1-12* and Columbia (Col) in response to plant growth regulators (PGRs), 1 µl of auxin (2,4-D), cytokinin (BAP), GA_3 or ACC at different concentrations were applied 2 days after the emasculation to each pistil, then the silique growth was determined after additional 5-7 days. Different PGRs with different amounts gave different effects on the silique. Results are presented below.

The response of wild type siliques to PGRs

The application of 2,4-D at levels of 0.1-10 nmols/ pistil to wild type Col did not result in apparent pistil elongation (Figure II-1, Figure II-2). Inversely, 1 nmol/ pistil 2,4-D inhibited the pistil development and 10 nmols/ pistil 2,4-D damaged the pistil and turned the pistil yellow and dried out, and then died (Figure II-1).

Moreover, application of different amounts (0.1-10 nmoles/ pistil) of GA_3 or BAP stimulated the Col fertilization-independent silique growth (Figure II-1, II-2). The extent of pistil elongation was dependent on the dosage applied. Higher amount application resulted in longer siliques. 0.1 – 10 nmol/ pistil GA_3 gave 2-3 folds of silique length as that without PGRs application (3 mm). Nonetheless, it was still shorter than that observed on the fertilized pistil that was approximately 14 mm (Figure II-1). The effect of BAP was weaker than that of GA_3. 1.0 – 10 nmol/ pistil BAP only generated one fold elongation of pistil (Figure II-1, II-2).

ACC applied to Col in low amounts (0.1-10 pmol/ pistil) also efficiently induced the pistil growth. 1.0 pmol/ pistil of ACC resulted in a double length of silique as compared with the control without PGRs application (Figure II-2).

The response of *axr1-12* siliques to PGRs

Compared with wild type Col, the auxin-resistance mutant *axr1-12* showed a different response of the pistil growth to 2,4-D. The application of 2,4-D at 0.1 nmols/ pistil cause the silique a double elongation as compared with the control. And the siliques also became thicker. However the treated pistils simultaneously turned slight yellow (Figure II-1, II-2). Low level of 2,4-D could stimulate the ovule growth slightly (Figure II-3). Increasing the levels of 2,4-D to 1 or 10 nmols/ pistil caused enhanced yellowing and

did not stimulated growth anymore. The 10 nmols/ pistil treatment made the pistil to become completely yellow, dried out and finally died. Results indicate that high concentration of 2,4-D is harmful for the silique development.

Treatment of *axr1-12* pistils with different amount (0.1-10 nmoles/ pistil) of GA_3 stimulated silique growth significantly and resulted in 2-3 folds elongation (Figure II-1, II-2). The silique length could be even comparable to that observed in fertilized *axr1-12* pistils, but no development was observed in the ovule after GA_3 treatment (Figure II-3).

BAP applied to *axr1-12* at levels of 0.1-10 nmols/ pistil and ACC at levels of 0.1-10 pmols/ pistil could not apparently influence the pistil growth (Figure II-1, Figure II-2).

Since different plant growth regulators have different effects on fertilization-independent silique development, different PGRs at different amounts, *e.g.* 0.1 nmols/ pistil of 2,4-D, 1.0 nmols/ pistil of GA_3, 1.0 nmols/ pistil of BAP, were then combined to analyse their effect on silique development (Figure II-4). The combination of GA_3 and 2,4-D stimulated the silique growth more effectively than the 2,4-D or GA_3 alone, and therefore a weak additive effect was showed between 2,4-D and GA_3. The addition of BAP counteracted the additive effect of 2,4-D and GA_3.
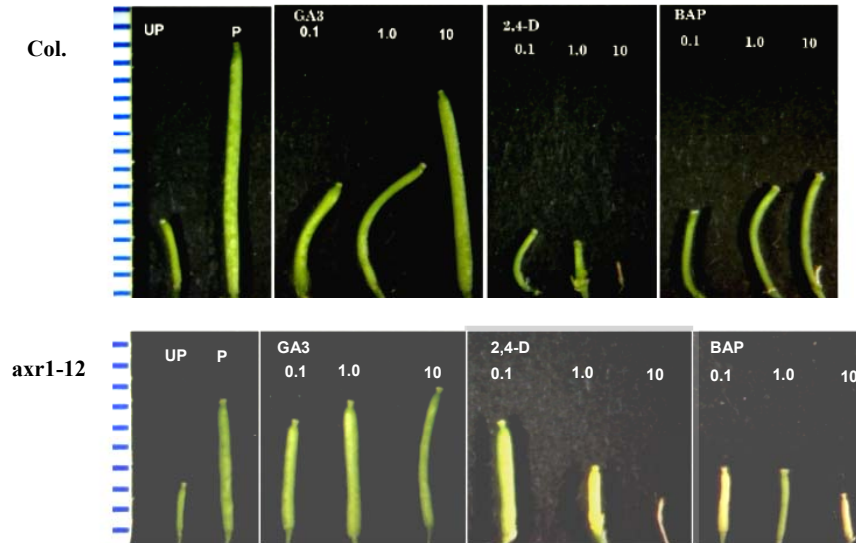
Figure II-1. Growth of siliques of wild type (Top) and the mutant *axr1-12* (Bottom) after different PGRs treatments. 1 µl of GA_3, 2,4-D or BAP was applied at different levels as indicated in each panel (nmoles/ pistil) to the pre-emasculated pistils. Silique growth was assayed seven days after the treatments. Scale on the left: 1 mm. UP: unpollination, P: pollination.

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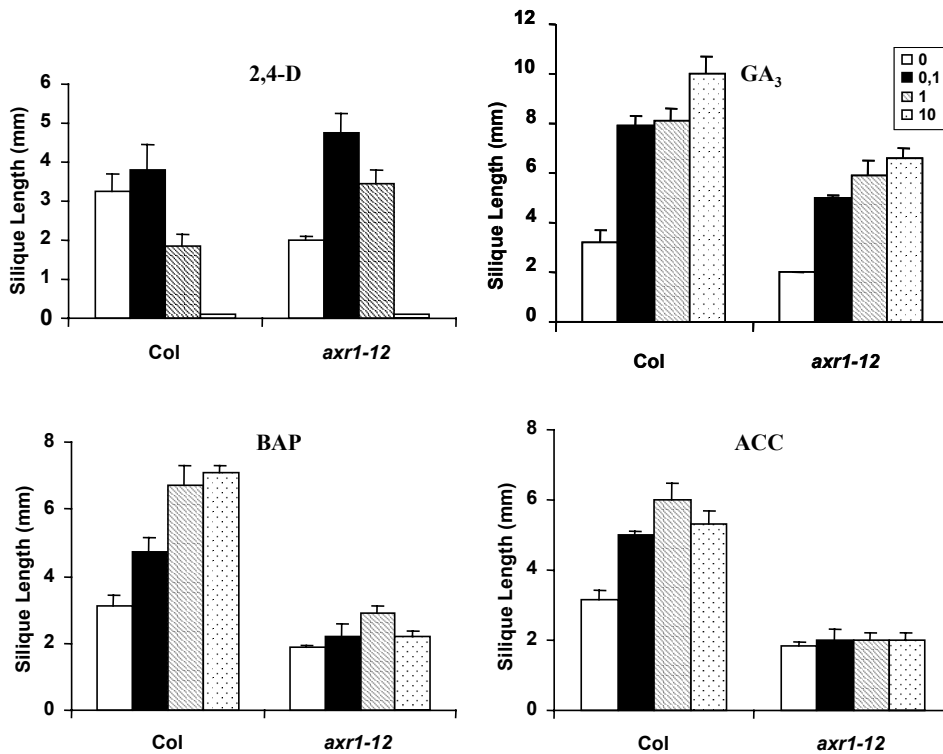
Figure II-2. Silique length of Col and *axr1-12* seven days after the the treament of emasculated pistils with different PGRs. 1 µl of PGRs was applied at different levels (0, 0.1, 1.0, 10 nmole/ pistil) to the pre-emasculated pistils. Silique growth was assayed five days after the treatments. The data is presented as the average (±SD) of 10 siliques

Figure II-3. Development of seed and silique of *axr1-12* ten days after treatment with 2,4-D (0.1 nmole/ pistil) or GA$_3$ (0.1 nmole/ pistil). The top panel shows the control siliques grew in nature and the emasculated pistil treated with H$_2$O. All pictures were in the same magnification.
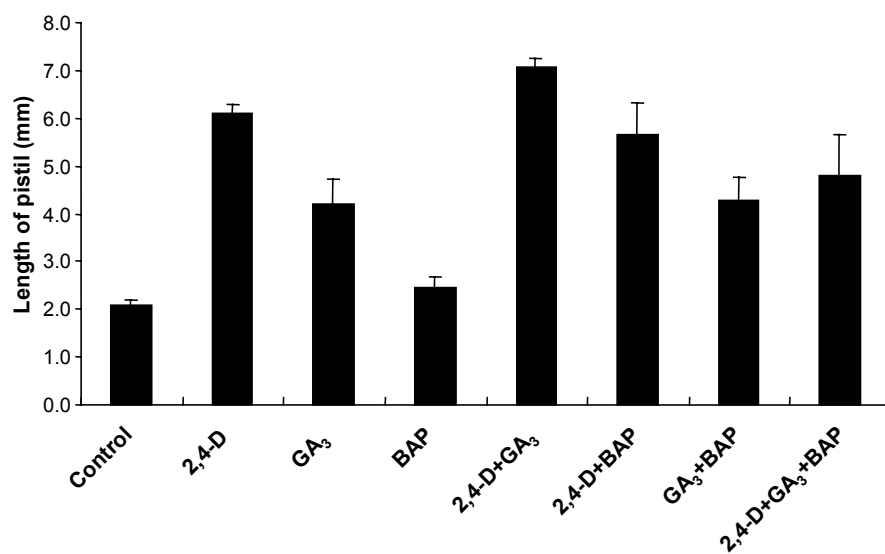
Figure II-4. Elongation of emasculated pistils of *axr1-12* seven days after treatment with different combinations of PGRs. 2,4-D, GA$_3$, and BAP was applied at 0.1 nmols, 1.0 nmols and 1.0 nmols/ pistil with 1 µl of droplet, respectively. The corresponding solution without growth regulators was used as the control. The data is presented as the average (±SD) of 10 siliques

Since *axr1-12* has very low fertility and seldom develops siliques, and some PGRs could induce the fertilization-independent silique development, different concentrations of 2,4-D and GA$_3$ were then sprayed on the flowering plants to induce the silique development. The purpose was to find a suitable PGR concentration for screening of the PGRs-sensitive mutants, and this concentration must not stimulate the silique development of the *axr1-12* but was expected to be able to stimulate the silique development of the PGRs-sensitive mutants.

The flowering *axr1-12* plants at 32 DAS (days after sowing) were sprayed with different PGRs buffered in 0.1 mM MES at different concentration once daily for 2 consecutive days, and the silique development was assayed 7 days later (Figure II-5). 0.1 mM 2,4-D induced
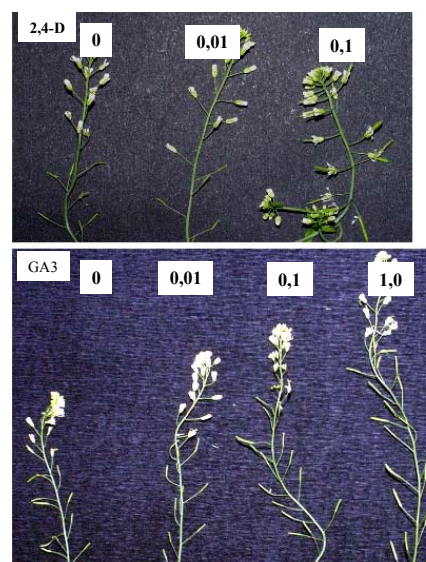
Figure II-5 Silique development of *axr1-12* after spraying of 2,4-D or GA$_3$. Plants at 32 DAS were sprayed two times at two consecutive days with 2,4-D or GA$_3$ in different concentrations (mM) as indicated and infloresceces were photographed seven days after the treatment.

silique growth effectively and made the siliques become thick and long, and 1.0 mM GA$_3$ stimulated the pistil elongation, while 10 times lower concentration, 0.01 mM 2,4-D and 0.1 mM GA$_3$, could not stimulate the silique development in *axr1-12*. These concentrations predicted to be able to stimulate the silique development in some mutants that enhanced the auxin signaling independent of *axr1*. Hence, 0.01 mM 2,4-D and 0.1 mM GA$_3$ were chosen to screen for auxin-hypersensitive or gibberellins-hypersensitive secondary mutants from the M2 population.

3.2 Isolation of suppressors from *axr1-12* in silique development

Mutant screen

Approximately 40,000 *axr1-12* seeds were mutagenized using 0.3% (v/v) EMS. These mutated seeds were sown on nutrient soil in a growth chamber and obtained about 30,000 M1 plants. In the M1 generation heterozygote chimeras for any given mutation should be contained (Glick and Thompson, 1993), therefore the mutants observed in this generation are probably the dominants. Progeny collected after selfing of M1 plants are referred to as the M2 generation, and should be segregating both heterozygotes and homozygotes for a given mutation. In M1 generation, 46 putants (putative mutants), which were able to clearly develop siliques at the later flowering stage (about 40 days after sowing (DAS)), were isolated. At maturity, those 46 putants were harvested separately. The remaining M1 plants, however, could also develop very few siliques with few seeds because of the cross-pollination, were mixture-harvested to generate the M2 seed pools. Seeds from about each 1000 M1 plants were collected to produce one M2 seed pool. 21 independent M2 seed pools were then derived and named as M2-1, M2-2, ……M2-21.

  The 46 putants were further checked for their ability to develop siliques in their M2 generation. Only 11 of them inherited the phenotype stably (Table II-1).

  In order to screen for suppressors of *axr1-12* that were more sensitive to 2,4-D or GA$_3$ in fruit development, 3,000 seeds from each M2 pool were sown on nutrient soil. The plants in 16 pools were treated with 0.01 mM 2,4-D and in 5 pools were treated with 0.1 mM GA$_3$ by PGR-spraying 2 times at an early flowering stage (at 31 and 32 DAS). 5 days after the treatment, the plants which developed siliques to a significant extent were considered as the auxin- or GA$_3$-sensitive candidates. 31 putants were thus isolated. All the isolated putants are summarized in Table II-1.

Table II-1. Suppressors screened from *axr1-12*.

Population (or pools)	Selection methods	Number of putant	Remarks
M1	-	23	Flowering 2 days earlier than Col and *axr1-12*
		31	
		32	Grew weakly, was left out
		40	
		43	Strong one
		46	
		48	
		52	
		53	
		58	
		59	
M2-1	2,4-D	201	
M2-3	2,4-D	202	
		203	
M2-5	2,4-D	204	
M2-6	2,4-D	205	
M2-9	2,4-D	210	
M2-11	2,4-D	206	
M2-15	2,4-D	207	
		208	
M2-21	2,4-D	209	
		211	
		212	
		213	
		214	Fruit petioles show epinasty
		215	
		216	
M2-20	2,4-D	220	Developed some parthenocarpic siliques, no seeds obtained
M2-16	2,4-D	221	
		222	
M2-18	2,4-D	223	
M2-14	2,4-D	224	
M2-19	GA$_3$	217	Bushy, higher than *axr1-12*
		219	Bushy, very short
M2-21	GA$_3$	218	Bushy
M2-13	GA$_3$	225	Parthenocarpy after sprayed with GA$_3$
		226	
M2-12	GA$_3$	227	
		228	
		229	

| M2-10 | GA$_3$ | 230 | Developed some parthenocarpic siliques, no seeds obtained |
| | | 231 | |

Developmental phenotype of the putative mutants

Figure II-6 shows the phenotypes of 3-weeks seedlings of some secondary putants and the control wild type Col and the original mutant *axr1-12* and. Rosette leaves of wild type expand plainly, the margins of it are round, and the petioles are long. Rosette leaves of *axr1-12* plants are irregular in shape and tend to curl downward seriously, the margins of leaves are slightly toothed, and the petioles are shorter than wild type leaves. In addition, the *axr1-12* plants are smaller than Col. Phenotypes of all secondary putants are just in the intermediate between Col and *axr1-12*. Their leaves are not curled down as much as the *axr1-12* but also do not expand as plain as the Col. Margins of leaves are toothed more slightly than *axr1-12*. The petioles are shorter than Col's but longer than *axr1-12*'s except Nr 219, a tiny plant. Hence, the shape of rosette leaves were affected by the *axr1-12* gene even in the suppressor plants.

The phenotypes of the adult plants of putants were also dramatically different from the original mutant *axr1-12*. Some of them had a phenotype very similar to wild-type Col (Figure II-7A-7E).

Based on the apical dominance, the silique and seed development, all putants were classified into 4 groups (Table II-2).

29 putants (Nr 31, 40, 43**,** 46, 48, 52, 53, 58, 59, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 212, 213, 214, 215, 216, 221, 222, 223, 228, 231) belong to group A that is characterized by a similar phenotype as the wild type Col plants. They were tall and exhibited apical dominance. They had completely developed siliques with partial or full seed setting. No seed-like structures were found in their siliques. The typical candidate in this group was Nr 43. It was directly screened from the M1 population. It had strong apical dominance, it could get a lot of siliques, and its fruit petioles showed epinasty (Figure II-7B). In their siliques, seeds were developed normally (Figure II-8). These traits were retained in the next several generations of selfing without segregation. Another typical candidate was Nr 214. It was selected from M2 by spraying with 2,4-D. It exhibited strong growth, apical dominance and had the siliques developed very well. Their petioles showed epinasty. However, in the next several generations of selfing or crossing with *axr1-12*, this putant exhibited an unstable phenotype: It had only partial seed setting in the siliques (Figure II-8), the siliques did not develop well any more and its fruit petioles did show epinasty.

9 candidates (Nr 23, 211, 217, 218, 224, 225, 226, 227, 229) were classified into group

B. They exerted the phenotype like plants in group A, presented apical dominance and developed siliques (Figure II-7C). In addition, they had another major character, that was, they developed siliques with seeds and/ or seed-like structures which had no embryo developed (Figure II-8, II-9). The typical one is Nr 211. It formed seeds and seed-like structures in a 1:1 ratio in siliques (Figure II-8). Another special one is Nr 225. It developed siliques after emasculating when it was sprayed with GA_3 in the M2 generation, it displayed somehow the parthenocarpic phenotype.

Nr 220 and Nr 230 were classified in the C group. These putants were characeried by a bushy appearance like *axr1-12*, but their shoot grew upright instead of the groveling behaviour of *axr1-12* (Figure II-7D). They developed siliques without seeds or seed-like structures (Figure II-8), so no seeds were obtained from these putants. In order to rescue these putants, the putants were crossed to wild-type Col and original mutant *axr1-12* as female or male parents. Unfortunately, no seeds were obtained from these crossings. The impossibility of rescuing the 2,4-D hypersensitive (Nr 220) and GA_3 hypersensitive (Nr 230) parthenocarpic mutants suggested that they carried the defects leading to lethality.

Nr 219 is a special line, it was classified in group D. The plant was short with very short internodes. It grew creepily and formed siliques abundantly (Figure II-7E). It developed siliques with seeds and seed-like structures (Figure II-8).

Table II-2. Classification of the mutants.

Group	Apical dominance	Seeds	Seed-like structures	Mutants
A	Yes	Yes	No	**Nr 43, 53, 214,*et al.***
B	Yes	Yes	Yes	**Nr 23, 211, 217, 218, 224, 225, 226, 227, 229**
C	No	No	No	**Nr 220, 230**
D	No	Yes	Yes	**Nr 219**

Figure II-6. Three-week-old seedlings of Col, *axr1-12* and some mutants.
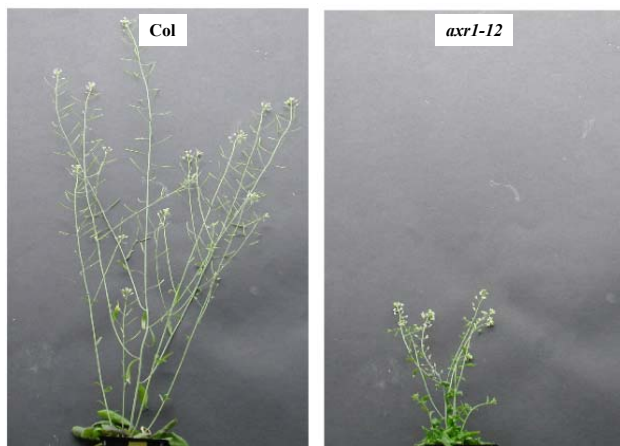
Figure II-7A. Morphology of adult wild type Col and *axr1-12* (45 DAS).

Figure II-7B. Morphology of adult mutants in group A (45 DAS).

Figure II-7C. Morphology of adult mutants in group B (45 DAS).

Figure II-7D. Morphology of Nr 230 in group C when it was isolated in M2 (50 DAS).

Figure II-7E. Morphology of adult mutant Nr 219 in group D (45 DAS).

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Figure II-8. Seed and silique development in putants. Seed (S), seed-like structure (SL) and non-developed ovule (OV) are indicated.
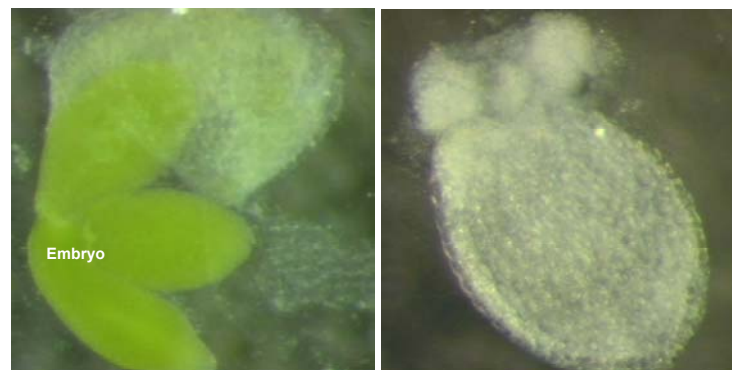
Figure II-9. Seed and seed-like structure. The seed coat of seed or seed-like structure was broken by applying a slight pressure, and then photographed under light microscope. Normal embryo was found in the seed (left) but not in the seed-like structure (right).

Do the mutants still carry the original mutation axr1-12?

The phenotypes showed that all putants suppressed the defect in silique development and most of them acquired the apical dominance again. They even recovered the auxin sensitivity (data will be shown later). However, it was still not clear if these putants were true suppressors of *axr1-12* or just were contamination of wild type Col. To clarify this problem, the *AXR1* specific CAPS (Cleaved Amplified Polymorphic Sequences) was established. The *axr1-12* (C→T) created a novel *Dra*I cleaveage site. In a 590 bp-genomic DNA fragment covering the mutated site (Figure II-10), additional *Dra*I cleavage site is already presented in wild type Col. Therefore cleavage of this fragment by *Dra*I will generate 2 fragments (167 bp and 423 bp) from wild-type Col, and 3 fragments (167 bp, 278 bp and 145 bp) from *axr1-12*. Thus, the *Dra*I recognizing sites can be used as an *AXR1* specific CAPS marker. The correct suppressors of *axr1-12* should have the same CAPS as *axr1-12*. Otherwise they are the contaminations.

```
75841  AACAGAGTTA GTGCACTTGC CTCTATGGCA CTGAAATTCA CCCTCAGTCT ATTTTTCACA
                 ──────────────────────────────▶
                         Forward primer

75901  TTGGTTATGT AGCTGTATGG TTTCTCTTTG CAGACACTAT ATCAATTTGC AGAAAATCTA
                                                                    ↓DraI
75961  TTTAGCCAAA GCCGAGGCTG ATTTTCTTGT CATTGAGGAA CGAGTTAAAA ACATTTTAAA

76021  GAAAATCGGT CGAGATCCGA GCAGCATCCC AAAACCAACA ATCAAGAGCT TCTGCAAGAA

76081  TGCAAGGAAA CTTAAAGTGA GTATACTAAA CTTCCTAACA TGTTTAATTT GTTTTCTAAT

76141  GTCAACGTTT TACCTATTGC TTGTTTACTT TTTCTTCGGA GCATTATCAG AAACATATGA

76201  TTTGAGTAAA GCATAAACCA TAATCCGTCC TTTGTAGTTG TGCAGATATC GTATGGTAGA

                                                  (↓DraI)
76261  GGACGAGTTC AGAAATCCTT CTGTAACTGA AATTCAAAAG TATTTAGCGG ACGAGGATTA
                                                     (T)

76321  CAGGTGCGAA TTTTCCCTTT TCATATGAGT ACAAAAACAT CAGATGATTT GAAAGATGAC

76381  CTTTCTACAA AATGTTCACT CTTTTGTCTG TCTCATACAT GCCATAAGAT GTTGTGATTG
                                                   ◀──────────────────
                                                         Reverse primer
```

Figure II-10. The sequences of a fragment in *AXR1* gene (from *Arabidopsis thaliana* chromosome 1 YAC yUP8H12). In this fragment one *Dra*I cleavage site is found in wild type Col. *axr1-12* mutation (C → T) generated another *Dra*I cleavage site. The primers for PCR-amplification, and the DraI recognizing sites are indicated.

Figure II-11 showed that all the tested putants exhibited the same *Dra*I digestion pattern as the *axr1-12*. It's clear that all these putants carried the *axr1-12* mutation rather

than were contaminant of wild type plants. All the other putants were also proven to carry the *axr1-12* mutation (data not shown).
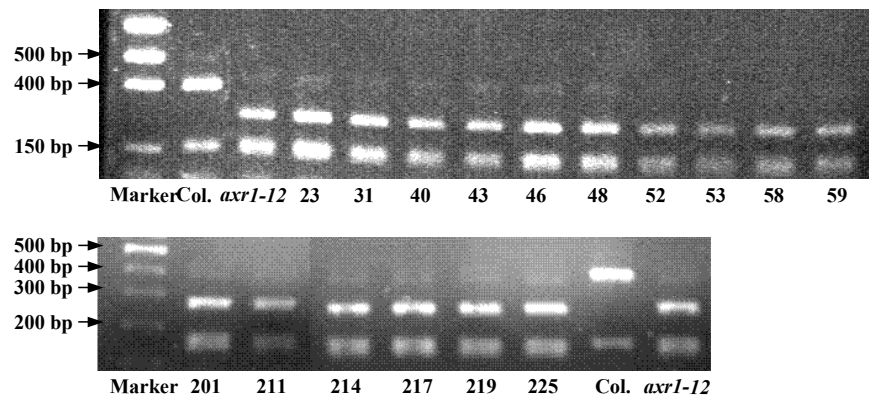
Figure II-11. Gel blots. The PCR products of wild-type Col, *axr1-12* and some putants were digested by *Dra*I then subjected to the agrose gel electrophoresis stained by ethidium bromide.

3.3 Root growth of suppressors in response to 2,4-D

Mutant *axr1-12* is an auxin resistance mutant. Seedlings of it were resistant to approximately 50-fold higher concentration of 2,4-D than wild type (Estelle and Somerville, 1987). To determine the root growth of its suppressors in response to 2,4-D, 4-days old seedlings were transferred to fresh MS medium containing different concentrations of 2,4-D and allowed to grow for another 3 days, and subsequently the root elongation was determined.

In PGR-free medium, the *axr1-12* and most of the suppressors showed little root elongation when compared with the wild type. Some suppressors, like Nr 31, Nr 40, Nr 48, Nr 211, Nr 214 and Nr 219 had even shorter roots compared to *axr1-12* (Figure II-12), suggesting that the new mutation in these suppressors might act upon root growth in an additive manner with the original *axr1-12* mutation. At any tested concentrations of 2,4-D, the root growth inhibition of *axr1-12* seedlings was less than that of wild-type seedlings. And most of the suppressors exhibited much more inhibition than the *axr1-12* but less than the wild type plants (Figure II-13A, Figure II-13B). At 0.3 µM of 2,4-D, the root growth of Col was inhibited completely (100%), while the root growth inhibition of *axr1-12* was only about 30%, and of suppressors was around 60-80%. At 0.03 µM, the root growth inhibition of most suppressors was around 30%, and of Col was 50%, while of the *axr1-12* was no more than 15% (Figure II-14). Few suppressors had different auxin response as others. They are Nr 31 and Nr 48, which showed less root growth inhibition than *axr1-12* at low concentration of 2,4-D (0.05 µM, 0.1 µM);
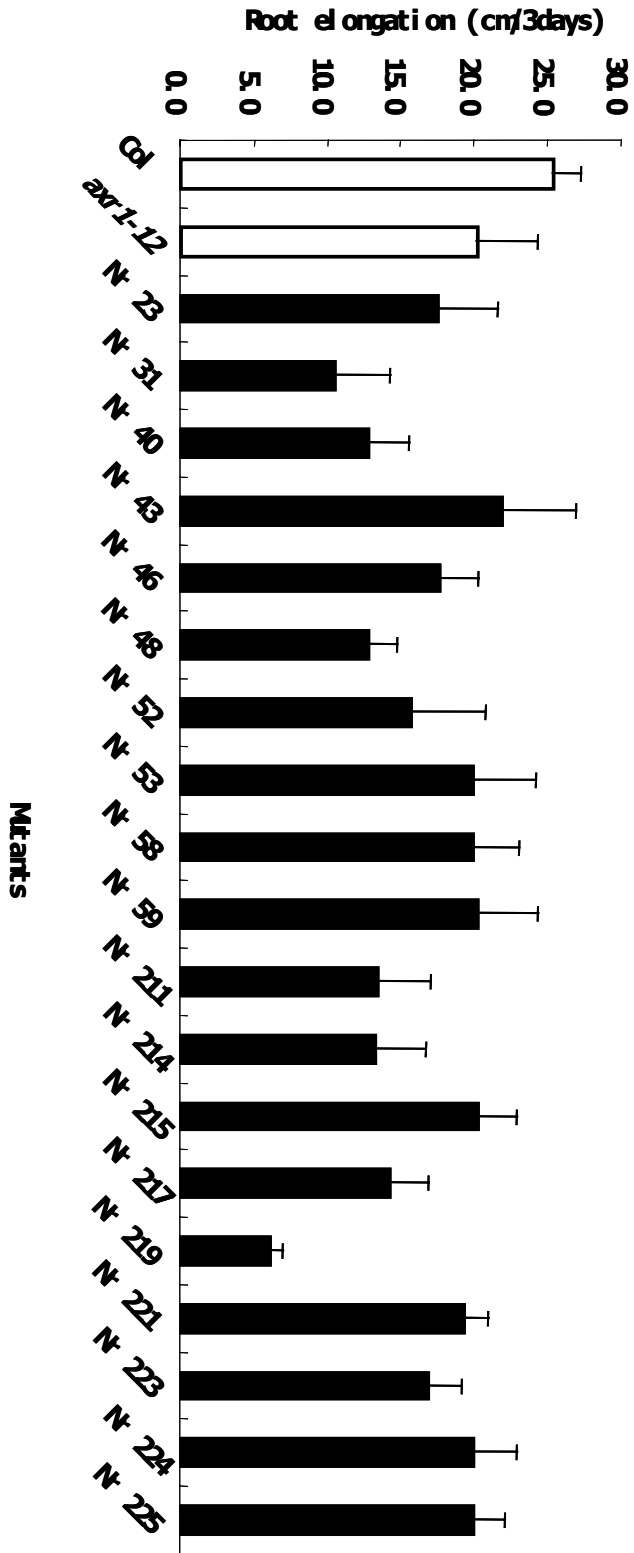
Figure II-12. Root elongation of the mutants and control seedlings (Col and *axr1-12*). 4 days-old seedlings were transferred onto fresh MS solid medium. Root growth within 3 days after transfer was determined (n=30, ±SD).
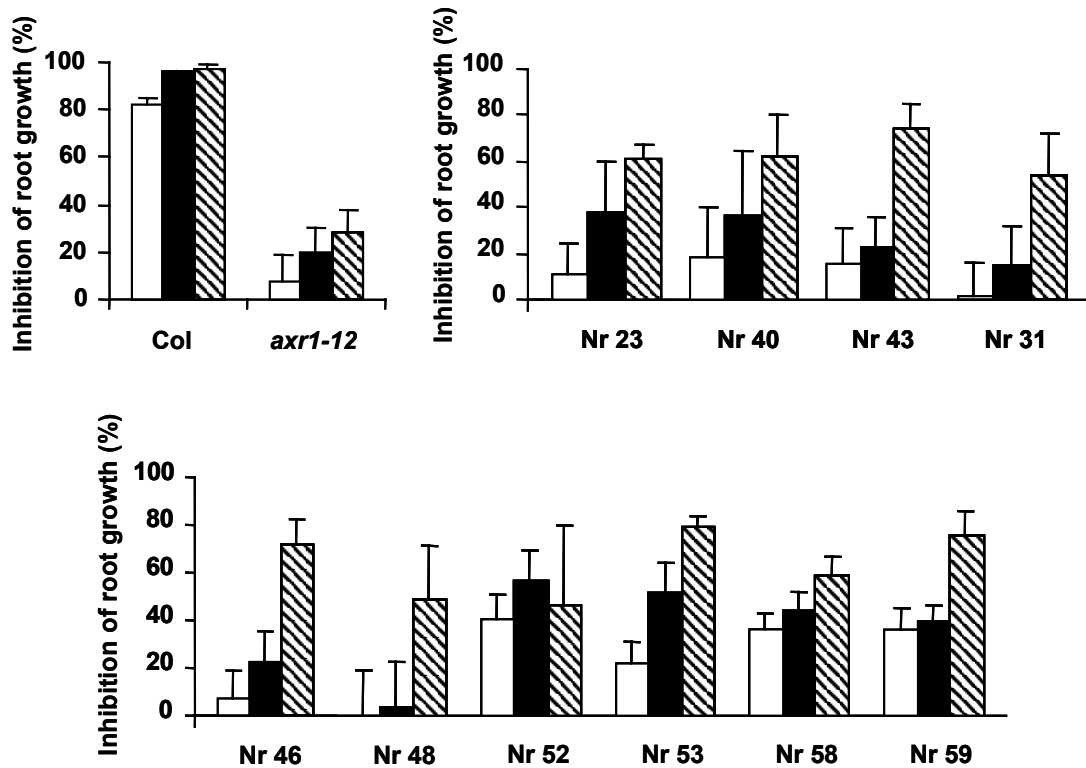
Figure II-13A. Root growth inhibition of control plants and mutants from M1 population. 4 days-old seedlings were transferred onto fresh MS solid medium with 2,4-D concentrations of 0.05, 0.1, 0.3 µM as indicated by open, solid and hatch bars, respectively. Root elongation within 3 days after transfer was determined (n> 15, ±SD). The root inhibition was calculated relative to the root elongation on the medium without 2,4-D (see Figure II-12).

Nr 221, Nr 224 and Nr 225, which revealed less root growth inhibition than *axr1-12* at 0.03 µM of 2,4-D. However, these mutants exhibited severer root inhibition than *axr1-12* at higher concentration of 2,4-D. It seems that these mutants have a very narrow threshold in response to auxin.

Root growth of most suppressors was inhibited more strongly than that of *axr1-12* at 0.3 µM 2,4-D but less than that of Col at 0.03 (or 0.05) µM. Hence, a modified definition is given here: *axr1-12* is defined as the **2,4-D insensitive plant.** If the root growth is inhibited more severe than that of *axr1-12* at 0.3 µM of 2,4-D, these plants are **2,4-D sensitive plants** (Col and all mutants are 2,4-D sensitive). And if the plant root growth is inhibited more severe than Col at 0.03 µM of 2,4-D, we call them **2,4-D hypersensitive** plants. This definition will be used in the following.
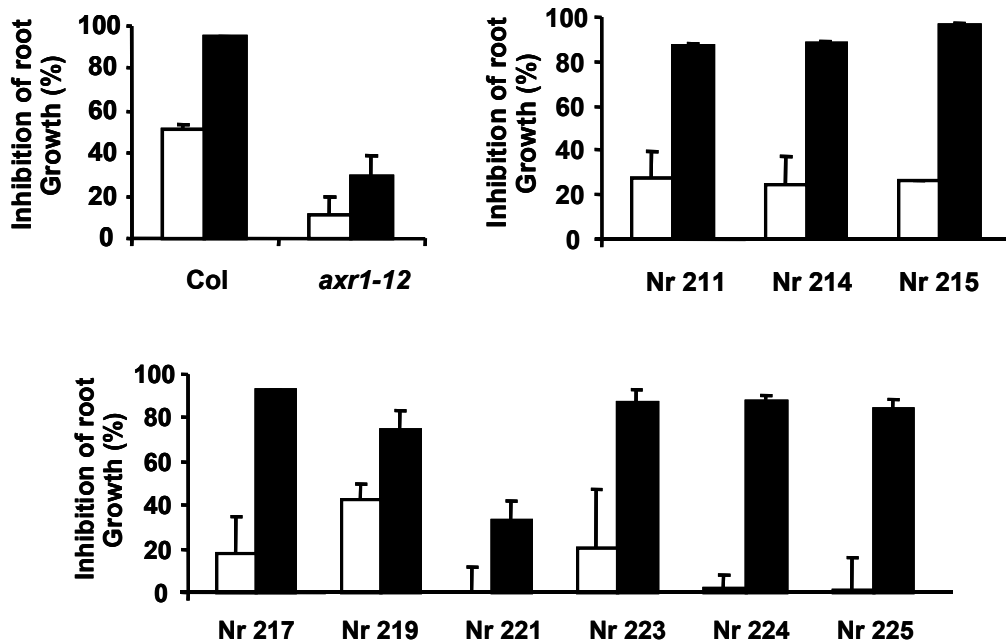
Figure II-13B. Root growth inhibition of control plants and mutants from M2 population. 4 days-old seedlings were transferred onto fresh MS solid medium plus 0.03, 0.3 µM of 2,4-D as indicated by open and solid bars, respectively. Root elongation within 3 days after transfer was determined (n> 15, ±SD). The root inhibition was calculated relative to the root elongation on medium without 2,4-D (see Figure II-12).
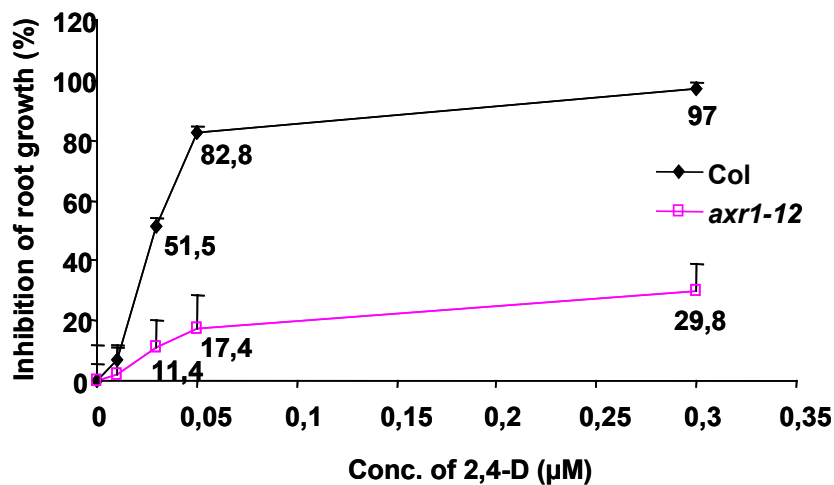
Figure II-14. Root growth inhibition of Col and *axr1-12*. 4 days old seedlings were transferred onto MS solid medium with various 2,4-D concentrations. Root growth within 3 days after transfer was determined (n=30, ±SD). The root inhibition was calculated relative to the root growth on medium without 2,4-D.

3.4   Other physiological characteristics of suppressor lines

Water loss and seed dormancy

Mutant Nr 225 of group B was isolated by spraying GA_3 in M2 generation. The adult plant of Nr 225 was as tall as Col and with plenty of siliques containing no seeds (Figure II-15), and its anthers were shorter than the pistil (data not shown). Its seeds and siliques developed very well after self-crossing by hand pollination (Figure II-15), demonstrating no major defects in the pistil and pollen development. Its pistils were able to develop into siliques after emasculating (Figure II-15), suggesting that it was probably a parthenocarpy mutant. Another special phenomenon was that this mutant displayed wilty phenotype when it was identified in M2 generation (data not shown), so it possibly carries a mutation affecting the ABA response.

It was reported that altered stomatal responses of ABA-insensitive or -hypersensitve mutants to ABA are mirrored in enhanced or reduced water loss of detached leaves, respectively (Meyer *et al.*, 1994; Pei *et al.*, 1998). To define if Nr 225 is an ABA-reponse

Figure II-15. 2-month-old plant of Nr 225 in M2 generation. The siliques (magnified) developed very well after emasculation or self-crossing.

mutant, the water loss of detached leaves as well as the seed germination ability which is also affected by ABA (Taiz and Zeiger, 1998) were assayed. However, no difference in the water loss of detached leaves was detected among leaves of Nr 225, Col and *axr1-12* (Figure II-16). Thus, Nr 225 is not a true wilty mutant. The wilty phenomenon observed might be caused by other reasons, *e.g.* physical damage or infection.

To analyse the seed dormancy affected by the endogenous ABA, the germination ratio of the matured seeds 0 to 4 weeks after harvest were assayed by germinating on MS medium for 5 days. The results showed that the immediately harvested seeds of either Nr 225, *axr1-12* or Col were unable to germinate and nearly all the seeds 4 weeks after

harvest could germinate. After harvest 1 to 3 weeks, the seed germination ratio of Nr 225 was higher than that of *axr1-12* and lower than that of the Col (Figure II-17).
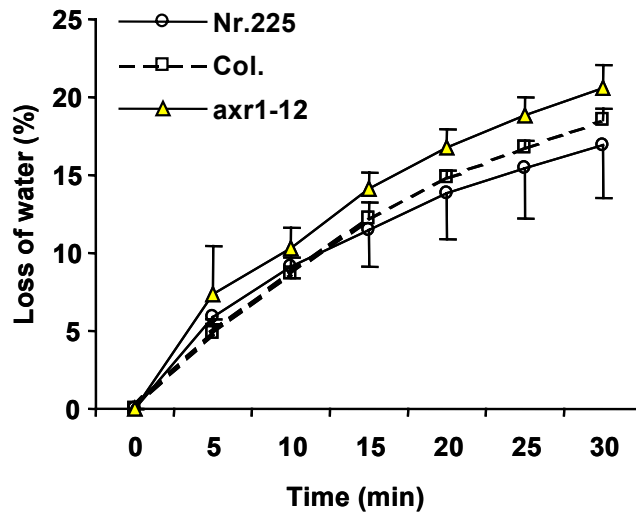
Figure II-16. Stomatal response measured by water loss of excised leaves. Three leaves of a comparable developed stage from 4-week-old plants (n=5) were measured at ambient conditions.
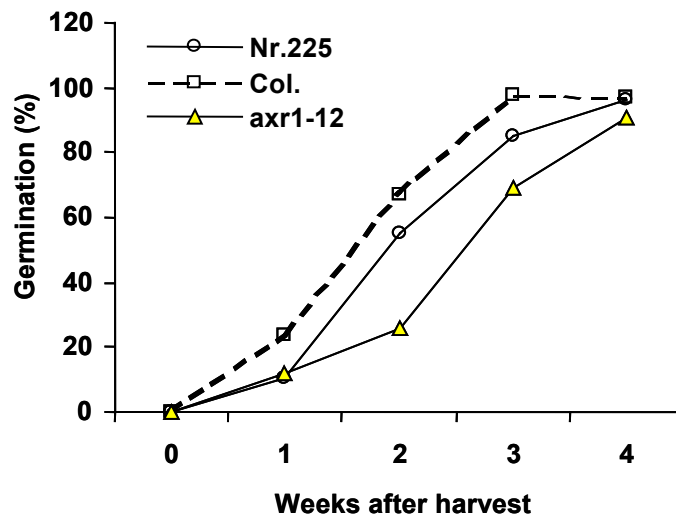
Figure II-17. Test of seed dormancy of Nr 225 and the control Col and *axr1-12*. A total of 100 seeds of each type at different time after harvest were germinated for 5 days on MS medium then the germination ratio was determined.
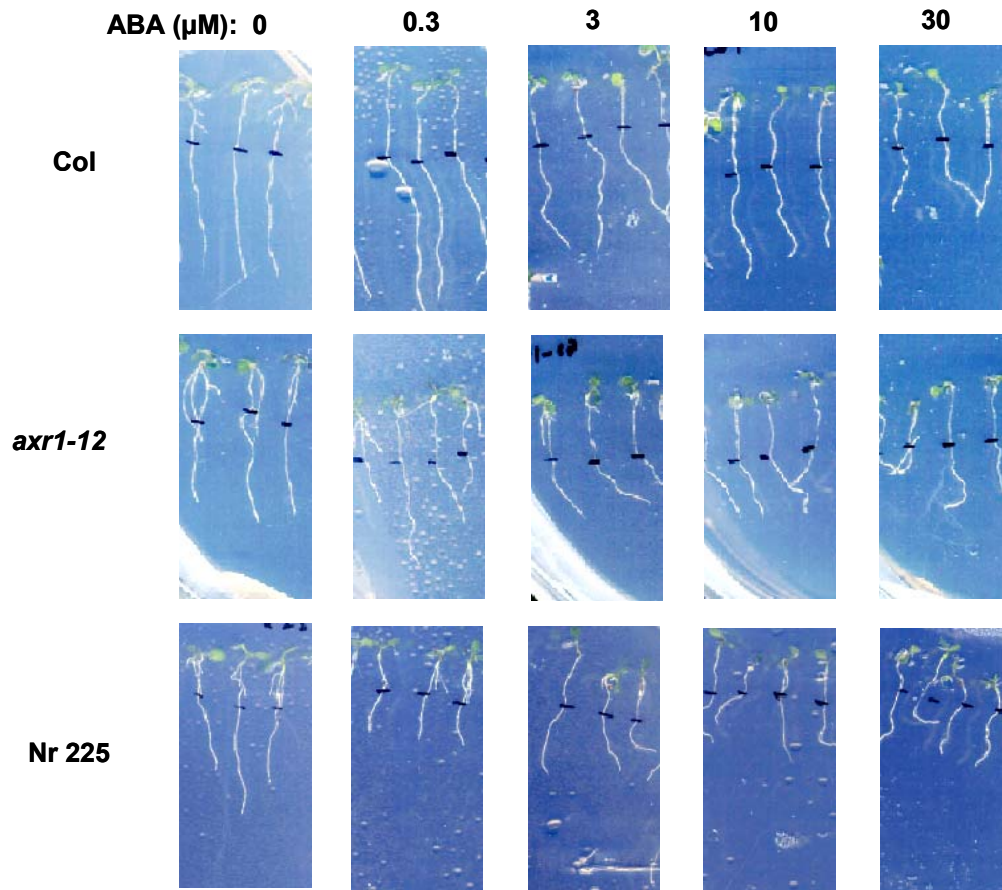
Figure II-18. The response of root growth of Nr 225 and control seedlings to ABA. 4-day-old seedlings were cultivated on MS medium in the presence of different ABA concentrations for additional 3 days. The root elongation is indicated by the black mark to the root tip prior to these three days.

Further analysis of the root growth response to ABA demonstrated no obvious difference among the Nr 225, Col and *axr1-12* (Figure II-18). Thus, these results did not convince us that Nr 225 was an ABA-response mutant.

Apical hook formation and root gravitropism

It was reported that *axr1-12* seedlings grown in the dark were unable to form an apical hook (Lehman *et al.*, 1996). The mutants Nr 214 of group A, Nr 23, Nr 211 and Nr 225 of group B were tested for apical hook formation in the dark. 4 days old dark-grown seedlings of Col, Nr 23, Nr 214 and Nr 225 formed an apical hook, while no apical hook was formed in *axr1-12* and Nr 211. Results indicated that the new mutations in Nr 23,

Nr 214 and Nr 225 were able to suppress this defect in *axr1-12* (Figure II-19).

Roots of *axr1-12* seedlings exhibited a slower response to gravity stimulus than the wild type Col roots (Lincoln *et al.*, 1990). This phenomenon was also observed in this experiment (Figure II-19). Roots of dark-grown Col seedling grew vertically. Roots of Nr 225 grew approximately vertically. While roots of *axr1-12* and the other mutants, especially the Nr 214, grew in rather random direction. It seems that these suppressors are unable to suppress the defect in gravitropism of *axr1-12*.

Lincoln *et al* (1990) reported that the *axr1-12* mutation reduced the height of dark-grown hypocotyls. However, no apparent difference of hypocotyls elongation among mutants, Col and *axr1-12* was observed in this experiment (Figure II-19).
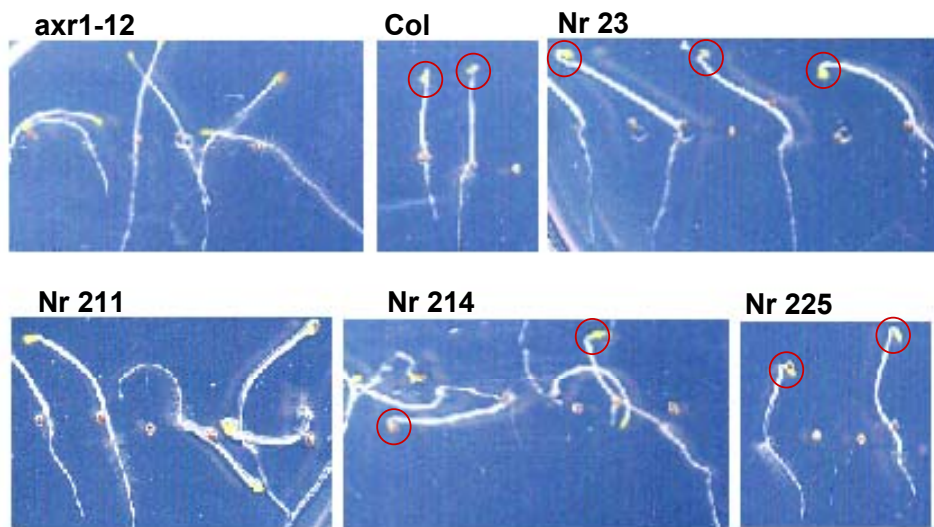
Figure II-19. 4 days old dark-grown seedlings of Nr 23, Nr 211, Nr 214, Nr 225, axr1-12 and Col. Apical hook is found in Nr 23, Nr 214, Nr 225 and wild type Col. seedlings. Nr 225 displays slight root gravitropism.

3.5 Genetic analysis of the suppressor lines

Genetic segregation

To analyze the genetic basis for suppressors of the *axr1-12*, each of the mutants was backcrossed to original mutant *axr1-12* by using the novel mutant as the female parent. The auxin-response of the root elongation in F1 and F2 generation from their crosses was determined.

F1 seedlings from the crosses of Nr 43, Nr 53 (of group A), Nr 23, Nr 211, Nr 224 and

Nr 225 (of group B) crossed *axr1-12* showed nearly equal root growth to their parents on the medium without 2,4-D. When grew on the medium containing 0.3µM 2,4-D, the root elongation of the mutants was reduced apparently, while the root elongation of the F1 seedlings was less affected by 2,4-D just like the *axr1-12* (Figure II-20), indicating that F1 seedlings were insensitive to 2,4-D. These results suggested that the suppressors were due to recessive mutations. The 3:1 segregation rate of auxin resistance in each F2 population further confirmed the recessive character of these mutants (Table II-3).

Nr 219 of group D was a tiny mutant with short root (Figure II-12) and short shoot (Figure II-7-5). When it grew on the medium containing 0.3 µM 2,4-D, the root elongation of the F1 seedlings from the cross of Nr 219 with *axr1-12* was apparently longer than that of Nr 219 but shorter than that of *axr1-12*. When the F1 seedlings grew on the MS medium without 2,4-D, the root elongation was nearly comparable with that of *axr1-12*. It indicated that the defect in root growth of Nr 219 suppressor had disappeared in its F1 seedlings (Figure II-20). Thus, this mutant is probably a recessive mutant too.

Nr 214 was possibly carrying a co-dominant mutation. There was no difference among the root growth of the F1 and the parents' seedlings grown on the MS medium in the absence of 2,4-D. When grown on the medium containing 0.3 µM 2,4-D, the Nr 214 seedlings had a very short root elongation and showed strong root growth inhibition, whereas no response from *axr1-12*. However, the root elongation of F1 seedlings was shorter than that of *axr1-12* but longer than that of the Nr 214 (Figure II-20). The F2 population was further analysed for the root response to 2,4-D through assaying of the root elongation in the presence of 0.3 µM of 2,4-D. Among the 42 seedlings in F2, 8 seedlings were resistant to 2,4-D, 9 were sensitive to 2,4-D as their parent Nr 214, and 25 just exhibited an intermediate response ---- were more sensitive than *axr1-12* and more resistant than Nr 214. This segregation ratio of 1:2:1 (Table II-3) revealed that Nr 214 probably carryies a semi-dominant mutation.
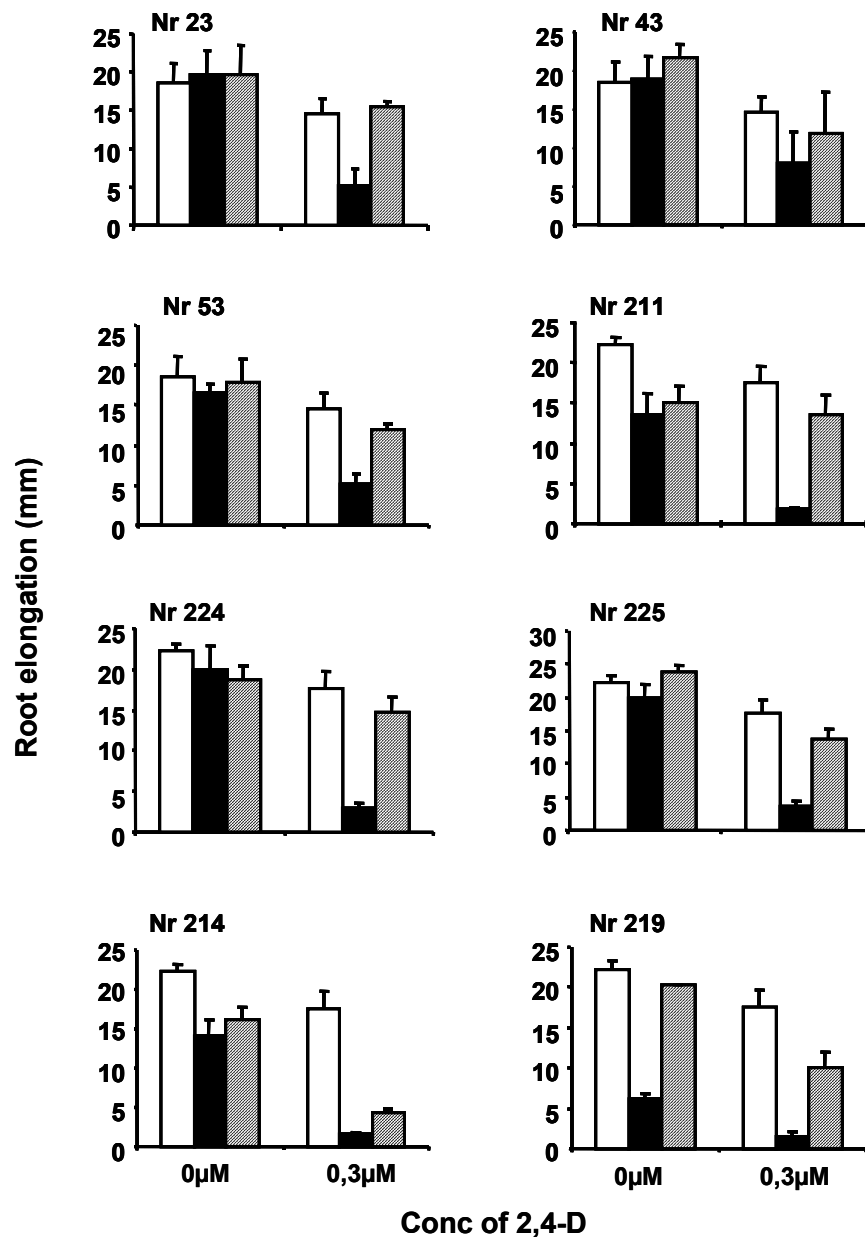
Figure II-20. Root elongation of *axr1-12*, novel mutants and F1 from crosses of *axr1-12* with novel mutants. 4 days old seedlings pre-cultivated on MS medium were transferred onto MS medium in the presence (0.3µM) or absence of 2,4-D. Root elongation within 3 days after transfer was determined (n > 15, ±SD). The root elongation of *axr1-12*, novel mutant and the F1 of *axr1-12*Xmutant are indicated by open, solid and hatch columns, respectively.

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Table II-3. Genetic segregation of 2,4-D sensitivity in some mutant lines. The phenotype was defined by the root growth response to 0.3 µM 2,4-D. a: x^2 calculation for an expected segregation of 3 insensitive to 1 sensitive. Critical x^2= 3.84. b: x^2 calculation for an expected segregation of 1 insensitive: 2 middle phenotype: 1 sensitive. Critical x^2= 5.99

Mutants	Number of plants							Conclusion for novel mutant
	Mutant	F1 of *axr1-12* X Mutant		F2 of *axr1-12*Xmutant				
	Sensitive	In-sensitive	Interme-diate response	In-sensitive (like *axr1-12*)	Interme-diate response	Sensitive (like the mutant)	X^2 (p> 0.05)	
Nr 23	20	11		30		11	0.008[a]	Recessive
Nr 43	20	6		26		8	0[a]	Recessive
Nr 53	20	7		23		8	0.011[a]	Recessive
Nr 211	20	8		11		3	0[a]	Recessive
Nr 225	20	8		29		10	0.009[a]	Recessive
Nr 214	20		5	8	25	9	1.514[b]	Semi-dominant

Linkage assay

In view of the above result concerning the root growth behavior on 0,3 µM 2,4-D, several recessive suppressors and 1 semi-dominant suppressor (Nr 214) were characterized. To determine if the suppressors carried the mutations at a locus extragenic of *AXR1*, these suppressors were crossed to wild type Col.

In the F1 population of the cross Col x mutant, the seedlings were sensitive but not hypersensitive to 2,4-D (Figure II-21), and the mature plants were tall and got siliques like Col. The explanation for this phenomenon is that the recessive mutation *axr1-12* has been complemented by the wild type *AXR1*. The segregation in F2 was further assayed.

Firstly, the segregation ratio in the F2 generation of the cross of suppressor with wild type Col was determined to give a hint of linkage between *axr1* and the suppressor gene.

If a recessive suppressor gene is linked to *AXR1* gene very tightly, the phenotype of the F2 generation will segregate at a ratio of 1 recessive suppressor to 3 wild type. If this suppressor is dominant, the segregation ratio in F2 will be 1 suppressor: 2 suppressor in wild type (*AXR1*) background: 1 wild type. Thus, no *axr1-12* phenotypic plant will

emerge in F2 generation if the suppressor gene is linked to *AXR1* gene, *e.g.* intragenic (Figure II-22).
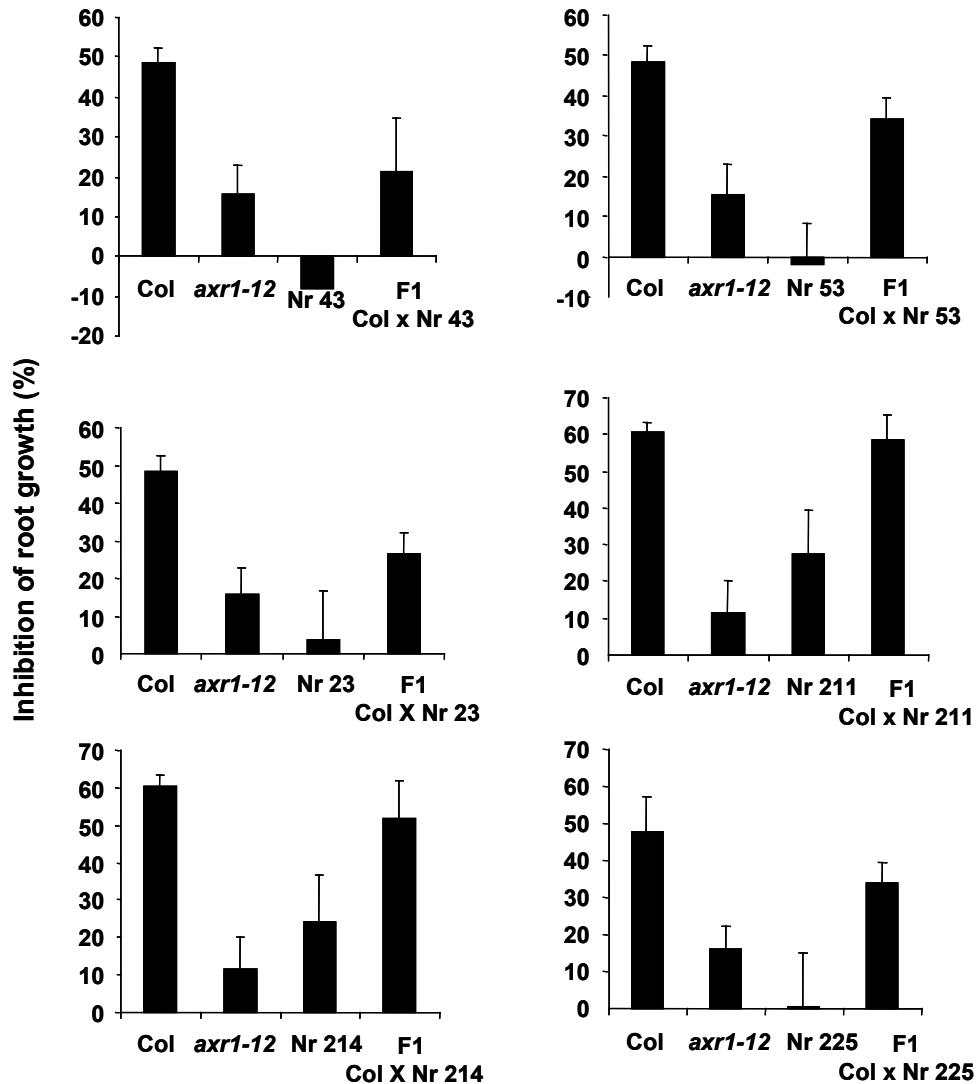
Figure II-21. Root growth of mutants and their F1 of the cross with wild type in response to 2,4-D. 4-day-old seedlings pre-cultivated on MS medium were transferred onto MS medium containing 0.03 µM 2,4-D. Root elongation within 3 days after transfer was determined (n> 15, ±SD). The root growth inhibition was calculated relative to the root growth on medium without 2,4-D.

If a recessive suppressor gene is not linked to the *AXR1* gene, the F2 generation of suppressor crossed to wild type Col will segregate at 1/16 suppressor (in *axr1-12* background), 3/16 *axr1-12*, 9/16 wild type and 3/16 suppressor in wild type (*AXR1*) background. The suppressor in wild type (*AXR1*) background might display the hypermoph phenotype which has the gene function more than the wild type level (Glick

and Thompson, 1993). If the suppressor carries a dominant mutation, the F2 generation will segregate at 1/16 *axr1-12*, 3/16 suppressor (in *axr1-12* background), 3/16 wild type and 9/16 suppressor in wild type (*AXR1*) background (hypermoph). Alternately, if the suppressor carries a co-dominant mutation, in addition to the *axr1-12* (1/16), suppressor (1/16), Col (3/16) and the likely hypermoph (3/16), some intermediate phenotypic progenies will emerge (Table II-4). In summary, if the suppressor gene is unlinked to *AXR1* gene, the *axr1-12* phenotypic plants will appear in F2 generation (Table II-4). Since the wild type Col (*AXR1*) is sensitive to auxin and the suppressors (in *axr1-12* background) are sensitive to auxin too, the phenotype of suppressors in wild type (*AXR1*) background is thus expected to be hypermorph and be hyper-sensitive to auxin.
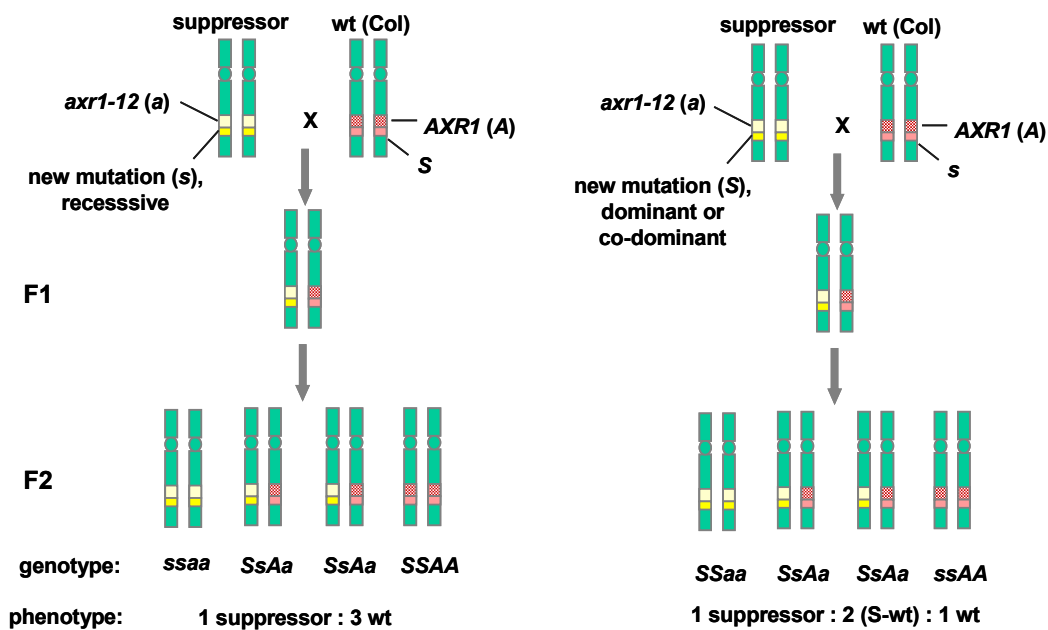
Figure II-22. The expected segregation of recessive, dominant or co-dominant suppressor trait tightly linked to *axr1-12* in a cross to wild type Col. The slight yellow, yellow, red hatch and pink bars indicate the loci of *axr1-12* (*a*), the suppressor gene (*s*-recessive, or *S*-dominant), the wild type *AXR1* gene (*A*) and the wild type suppressor gene (*S*-dominant, or *s*-recessive), respectively.

Table II-4. The expected Mendelian segregation of recessive, dominant and co-dominant suppressor trait un-linked to *axr1-12* when crossed to wild type Col. A: *AXR1*, a: *axr1-12*, S: dominant suppressor, s: recessive suppressor.

		segregation in F2					
If new mutation is recessive	genotype	1/16 *ssaa*	3/16 *S-aa*		3/16 *ssA-*		9/16 *S-A-*
	phenotype	1/16 Suppressor	3/16 *axr1-12*		3/16 Hypermorph?		9/16 Col
If new mutation is dominant	genotype	1/16 *ssaa*	3/16 *S-aa*		3/16 *ssA-*		9/16 *S-A-*
	phenotype	1/16 *axr1-12*	3/16 Suppressor		3/16 Col		9/16 Hypermorph
If new mutation is co-dominant	genotype	1/16 *ssaa*	2/16 *Ssaa*	1/16 *SSaa*	3/16 *ssA-*	6/16 *SsA-*	3/16 *SSA-*
	phenotype	1/16 *axr1-12*	2/16 intermediat of *axr1-12* and suppressor	1/16 suppressor	3/16 Col	6/16 intermediat of Col and hypermorph	3/16 hypermorph

Seedlings in *axr1-12* background or in *AXR1* background are easy to be distinguished by the morphology of leaves (Figure II-6, Figure II-23). Leaves of seedlings in *axr1-12* background were curled down, while leaves of seedlings in *AXR1* background expanded very well. For scoring the segregation of F2 generation of the cross suppressor x Col, 4-days old seedlings were preliminarily distinguished into two types by the leaf morphology. Seedlings of the first type were those *axr1-12* background seedlings (aa) which had down-curled leaves. They included *axr1-12* and suppressors. Seedlings of the second type were the *AXR1* background plants (AA(a)), including the wild type Col and the suppressor in *AXR1* background. Since that suppressors are more sensitive to 2,4-D than *axr1-12* plants and the suppressor in *AXR1* wild type background are most likely the hypermorph which might be more sensitive to 2,4-D than Col, F2 seedlings of the first type were transferred onto the medium containing 0.3 µM 2,4-D and of the second type were transferred onto medium containing 0.03 µM 2,4-D. They were allowed to grow for additional 3 days for root growth assay. The root growth of suppressors would be inhibited more seriously than that of *axr1-12* in the presence of 0.3 µM 2,4-D, and the suppressor in Col background are probably more sensitive to 0.03 µM 2,4-D than wild type Col plants. Thus, the *axr1-12* phenotypic plants, suppressors (in *axr1* background), wild type Col and those plants carrying suppressor mutation in Col background were distinguished.
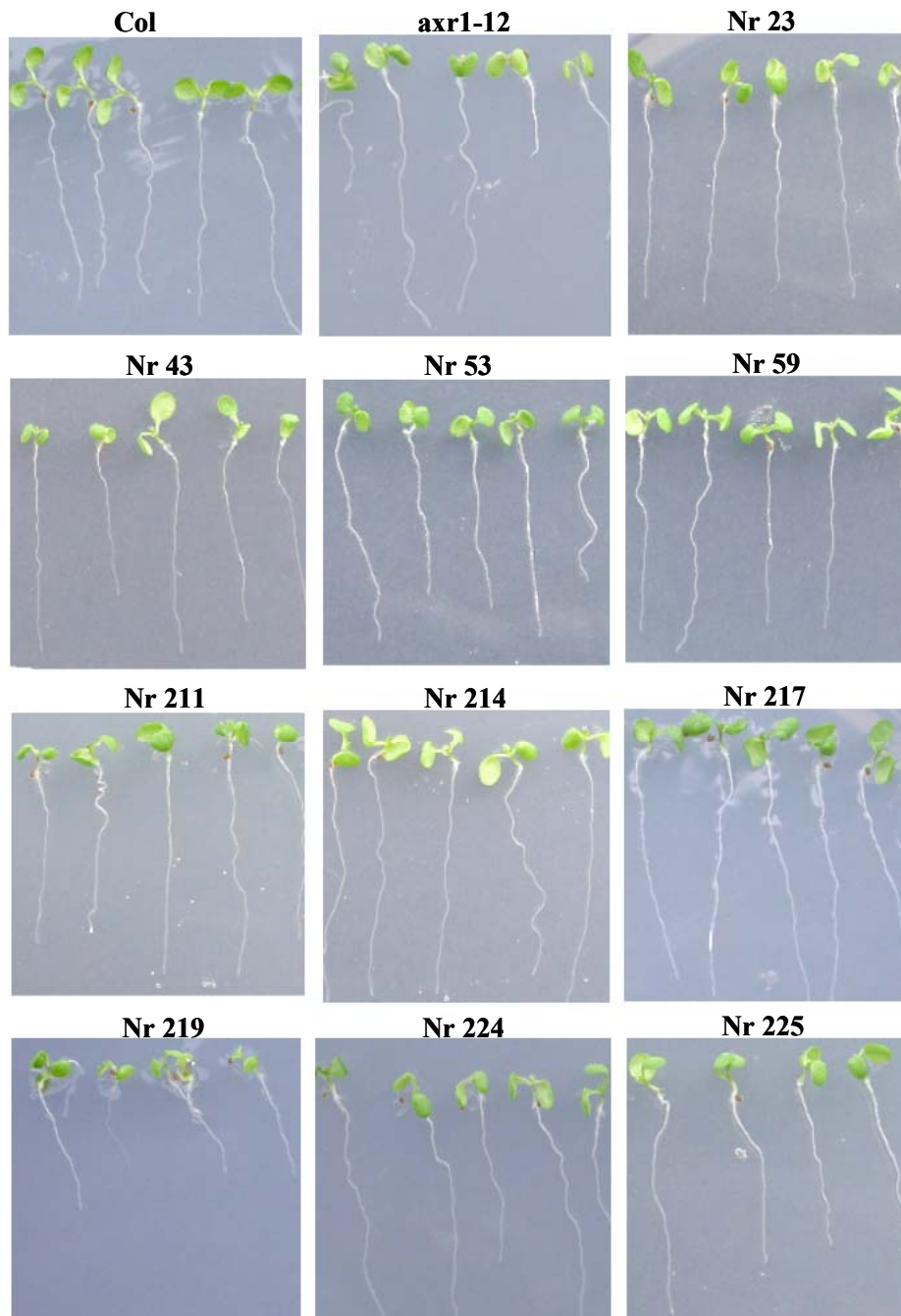
Figure II-23. Four days-old seedlings grown on MS medium. The leaves curling downwards were used to distinguish the *axr1* plants from *AXR1* plants.

Three mutants of group A (Nr 43, Nr 53 and Nr 214) and three mutants of group B (Nr 23, Nr 211 and Nr 225) were examined for the gene linkage to *AXR1* as mentioned

above according to the seedling's leaf morphology and root growth in response to auxin in F2. All these tested mutants are recessive mutants except the Nr 214, a co-dominant mutant. As shown in Table II-5, the *axr1-12* phenotypic plants emerged in the F2 progeny of all the tested crosses. Since the *axr1-12* phenotypic plants can only appear in the F2 progeny in the case of the suppressor trait unlinked to *AXR1* (Figure II-22; Table II-4), thus, all the tested suppressors are deduced to have a mutation unlinked to *AXR1*. In a population of 63 seedlings of F2 resulting from cross of Col x Nr 23, 9 *axr1-12* phenotypic plants, 5 suppressor phenotypic plants and 49 Col phenotypic plants were yielded. No auxin-hypersensitive plants appeared If the expected Col phenotypic and hypermorph plants were combined to conduct a chi-squared test, the result is well consistent with the expected segregation ratio of a single recessive suppressor trait unlinked to *axr1-12* (3 *axr1-12*: 1 suppressor: 12 (Col + hypersensitives)). Similar segregation ratio was also presented in the F2 of Col crossed with the recessive mutant Nr 43 and Nr 53 of group A, and with the recessive mutant Nr 211 and Nr 225 of group B (Table II-5). Results revealed that the Nr 23, Nr 43, Nr 53, Nr 211 and Nr 225 all carried a single recessive mutation unlinked to *axr1-12*. However, the expression of these new mutations was dependent on the original mutation *axr1-12* since no auxin-hypersensitives were obtained in the F2.

Of 90 F2 seedlings from the cross of the co-dominant mutant Nr 214 with Col, 25 were observed to display curling down leaves and 5 were *axr1-12* phenotypic plants (Table II-5). It indicates that the suppressor trait of Nr 214 is unlinked to *AXR1*. When F2 seedlings of Col x Nr 214 was assayed for the root elongation in the presence of 0.03 μM 2,4-D, 2-25 cm root elongation were observed. Among them 70% seedlings had the elongation of 2-12 cm and 30% had the elongation shorter than 7 cm. As the control, 100% of the wild type Col had 7-12 cm elongated roots and 100% of the *axr1-12* had 12-25 cm elongated roots (Figure II-24). Those seedlings with short root elongation (< 7 cm) were likely more sensitive to 2,4-D than the Col and thus were probably the hypermorph plants. The statistic analysis showed that the segregation of phenotypes in F2 of Nr 214 x Col was not consistent with the single dominant gene's segregation ratio. However, if a chi-squared test was performed following the combination of the plants which exhibited phenotypes of Col and auxin-hypersensitives, a low value of chi-squared is achieved corresponding to the expected ratio 1 (*axr1-12*) : 3 (suppressors) : 12 (Col + hypersensitives) (Table II-5). Furthermore, when the F2 seedlings grew up, the auxin-insensitive individuals exhibited the morphology of *axr1-12*, while the hypermorph ones resulted in strong, and tall adult plants like Col wild type but no other hypermorphic phenotype was observed. Results indicate that the Nr 214 carries a co-dominant mutation unlinked to *axr1-12*.

Table II-5. Genetic segregation of 2,4-D sensitivity of mutants when crossed to Col.

Cross	Segregation of phenotype in F2				$X^{2\ a}$
	axr1-12	suppressor	Col	hypersensitive	
Col X Nr 23	9	5	49	-	1.015[b]
Col X Nr 43	6	6	47	-	3.928[b]
Col X Nr 53	15	4	48	-	0.583[b]
Col X Nr 211	14	2	65	-	2.240[b]
Col X Nr 225	8	5	48	-	1.517[b]
Col X Nr 214	5 (20-21 cm)[e]	20 (12-20 cm) [e]	37	28	34.76[c]
			65 (2-12 cm) [e]		0.741[d]

a. probability > 0.05

b. Calculated based on an expected ratio of 3 axr1-12 : 1 suppressor : 12 (Col+hypersensitive) (critical x^2 = 5.99)

c. Calculated based on an expected ratio of 1 axr1-12 : 3 suppressor : 3 Col: 9 hypersen-sitive. (critical x^2 = 7.82)

d. Calculated based on an expected ratio of 1 axr1-12 : 3 suppressor : 12 (Col+hypersensitive) (critical x^2 = 5.99)

e. Root elongation in the presence of 0.03 µm 2,4-D
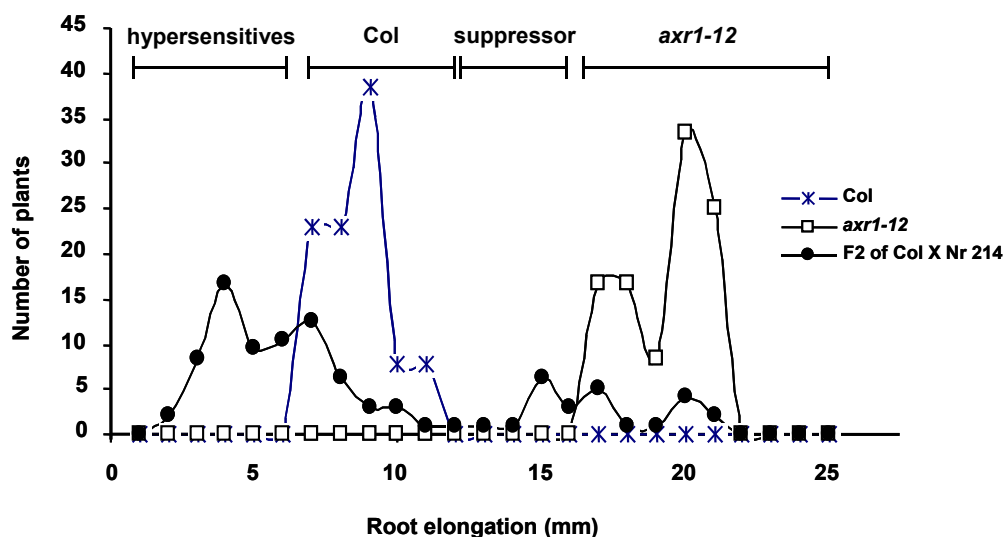
Figure II-24. Distribution of root elongation of segregating seedlings in the presence of auxin. 4 days old seedlings were transferred onto MS medium containing 0.03 µM 2,4-D and grown for another 3 days, the root elongation and the corresponding number of seedlings were recorded.

Backcrosses

Normally, any new mutant must be backcrossed to wild type for 3 to 5 times to clean up the genetic background (Glick and Thompson, 1993). In this experiment, the mutants were isolated from *axr1-12*, so they were backcrossed to *axr1-12*. The backcross was done in the following way: the first backcross was mutant x *axr1-12* to obtain B1, the second backcross was B1 x *axr1-12* to produce B2, B2 was then self-crossed to produce a new generation (S). The auxin hypersensitive individuals in this generation were then selected and used for a third backcross to *axr1-12* to generate B3. The individuals of B3 were self-crossed to obtain the first self-crossed generation (S1) of the three times backcross, and the 2,4-D sensitive plants in this generation were self-crossed to generate the next

Figure II-25. Flow chart of backcross.

Mutant X *axr1-12*   (backcross 1)

⇩

B1

⇩ **X *axr1-12***   (backcross 2)

B2

⇩ ⊗

S of B2 (selection of auxin sensitives)

⇩ **X *axr1-12***   (backcross 3)

B3

⇩ ⊗   **(selfing)**

S1

⇩ ⊗   **(selfing)**

S2

generation (S2) (Figure II-25). Auxin-response analysis showed that all the tested S2 seedlings of Nr 211, Nr 214, Nr 23, NR43, Nr 53, Nr 217 and Nr 225 were sensitive to 2,4-D, indicating that these suppressors were stably inherited. The Figure II-26 presents part of the analysis with the backcrossed progenies grown on the medium containing 0.3 μM 2,4-D.

   To obtain the mutant carrying the mutation from Nr 214 in wild type (*AXR1*) background, the Nr 214 was further crossed to Col. This cross yielded some auxin hypersensitive F2 plants in the progeny which were considered more sensitive to auxin based on the shorter root elongation at 0.03 μM 2,4-D than their parents (Figure II-24, Figure II-27). To isolate the auxin-hypersensitive individuals that were carrying the homozygous *AXR1* gene, the putative auxin hypersensitive seedlings were allowed to grow up and checked by the *AXR1*-CAPS marker (Figure II-10). 2 bands (167 bp and 423 bp) and 4 bands (167 bp, 145 bp, 278 bp and 423 bp, Figure II-28) were expected to be visualized respectively in the gel blots of the *Dra*I-digested PCR products of the *AXR1/AXR1* homozygotes and the *AXR1/axr1* heterozygotes. The selected *AXR1*

axr1-12   Nr 211   Nr 214

S2 of 3 times
backcrosses of
Nr 211 X *axr1-12*

S2 of 3 times
backcrosses of
Nr 214 X *axr1-12*

Figure II-26. The 2,4-D response of mutants and their offsprings. 4-days old seedlings were transferred from MS medium onto MS medium plus 0.3 µM 2,4-D and grown for additional 3 days. The root elongation during these three days is indicated by the black mark to the root tip prior these three days.

homozygous individuals should carry the homozygous (SS) or heterozygous (Ss) suppressor mutation (Table II-4), their offspring (F3) should be 100% or 75% hypersensitive to auxin, respectively. However, there were only few individuals in F3 exhibited the auxin-hypersensitivity as obtained in the previous generation, most of them exhibited slight inhibited root growth at 0.03 µM 2,4-D (Figure II-27). A special phenomenon occurred in the F4 generation. Only about 50% seeds could germinate. It seems not because of the seed dormancy since the seeds 1 month after harvest still got the comparable germination ratio. It is likely the mutation caused defects in seed development, *e.g.* stagnation of the embryo development. When the F4 seedlings were transferred to the medium containing 0.03 µM 2,4-D, some individuals exhibited auxin-hypersensitive. Unfortunately, the growth behavior of individuals in this generation was no long consentaneous (Figure II-27). This phenomenon inevitably set the difficulty to the gene mapping.
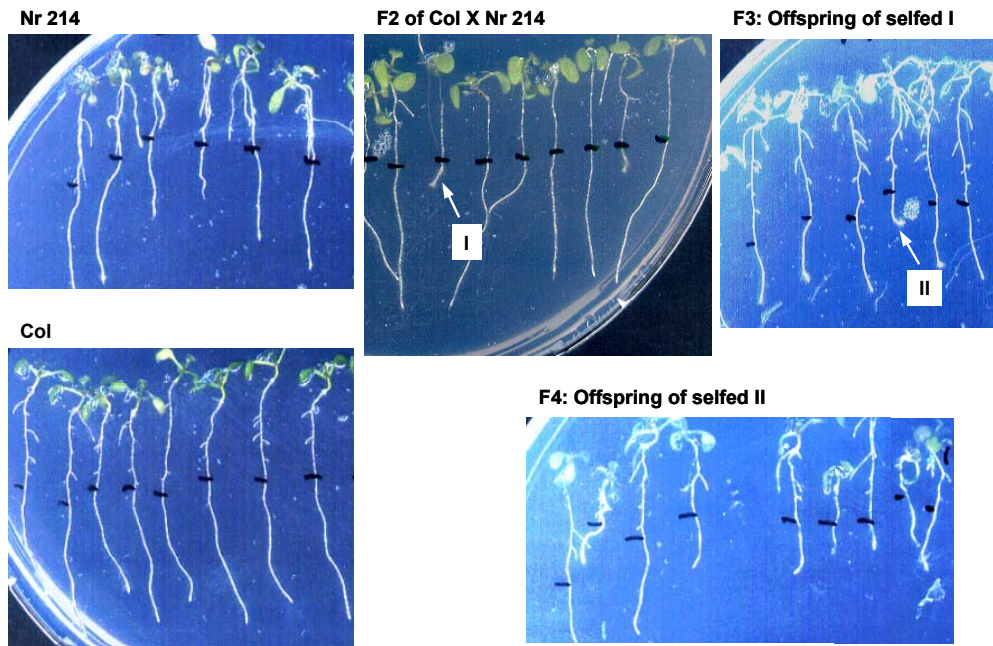
Figure II-27. 2,4-D response of Nr 214 and its offsprings. 4-day-old seedlings were treated for 3 days on MS medium containing 0.03 µM 2,4-D and the root elongation was recorded. The black mark was made to the root tip prior the 3 days incubation.
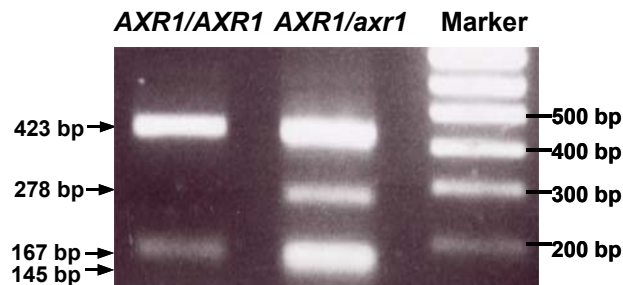
Figure II-28. The gel blots of the *DraI*-digested PCR products of homozygote *AXR1/AXR1* and heterozygote *AXR1/axr1* F2 plants.

Allelic Test

To determine if mutants carry mutations at the same locus, crosses between mutants were performed. Results showed that the F1 seedlings of Nr 53 x Nr 43 were sensitive to auxin as their recessive parents were (Figure II-29), and the morphology of all the adult F1 plants were also similar to their parents (data not shown), indicating that Nr 43 and Nr 53 carry allelic mutations. The F1 seedlings of Nr 23 x Nr 43 and Nr 23 x Nr 53 were less sensitive to auxin than their parents but more sensitive than the original mutant

axr1-12 (Figure II-29), suggesting that Nr 23 carries a mutation at a distinct locus as that of Nr 43 and Nr 53.
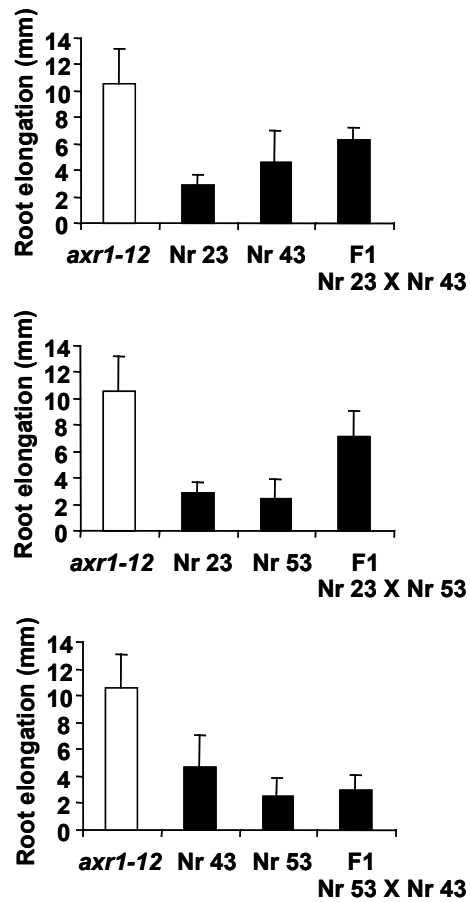
Figure II-29. Root elongation of mutants and their offspring in response to 2,4-D. 4 days old seedlings were transferred from MS onto MS containing 0.3 µM 2,4-D, the root elongation within 3 days after transfer was determined (n> 15, ±SD).

4   Discussion

4.1   Plant hormone and fruit development

In general, fruit development is initially stimulated by the fertilization and seed development, following them are the early fruit development phases: (I) fruit set, (II) a period of rapid cell division, (III) a cell expansion phase (Gillapsy, *et al.*, 1993). Plant hormones play crucial role in regulating the process of these three phases.

Gibberellins are generally assumed that to be necessary to trigger fruit set and to stimulate cell division as well as to maintain cell expansion. It usually acts co-ordinately with auxin and the other hormones. Gibberellins produced from the growing pollen and the following elevated auxin produced by the style and the ovary are believed to be the positive signals that triggers the fruit set and development (Gustafson, 1960; Gillapsy, *et al.*, 1993). GA_3 was reported to stimulate cell division of the mesocarp and endocarp in *Arabidopsis* (Vivian-Smith and Koltunow, 1999). In the phase III, fruit growth is mostly due to an increase in cell volume. It is generally accepted that auxins are responsible for the increase in cell expansion in fruit tissue (Rayle and Cleland, 1992; Vivian-Smith and Koltunow, 1999). However, the peak of gibberellin accumulation is found to coincide with cell expansion in this phase. In tomato, GA_3 has been observed to induce mesocarp cell expansion with restricted cellular division, while auxin treatment stimulated cell division (Bünger-Kibler and Bangerth, 1982). In this work the PGRs application experiment demonstrated that 2,4-D could induce the siliques of *axr1-12* to grow and elongate, and GA_3 stimulated the siliques of both wild type Col and *axr1-12* to elongate (Figure II-1, Figure II-2). The results revealed a positive regulation of gibberellins and auxins on fruit development. The additive effect of GA_3 and 2,4-D demonstrated in this work (Figure II-4) might be the result of the cell expansion induced by auxin and cell elongation and division induced by GA_3 (Vivian-Smith and Koltunow, 1999).

The siliques of Col treated with 2,4-D were yellow and finally died (Figure II-1). This may be because the concentration of 0.1-10 nmoles 2,4-D are too high and toxic to the Col siliques. Alternately, the auxin at high level stimulates ethylene production (Woeste *et al.*, 1999) and promotes senescence of siliques. For the auxin insensitive mutant *axr1-12*, 0.1 nmole 2,4-D is still possible to induce growth of silique but 1-10 nmoles 2,4-D lead to silique yellowing. The results indicate that the *axr1-12* requires auxin at least 10 times more to show an equal effect as in Col. The experiment of PGRs-spraying showed that 0.1 mM 2,4-D or 1 mM GA_3 could induce the axr1-12 siliques to grow. Hence, 10 times lower concentration of 2,4-D (0.01 mM) or GA_3 (0.1 mM) might be able to induce those plants, which are more sensitive to auxin or gibberellins than

axr1-12, to develop the siliques. Based on this assumption, some mutants (*e.g.* Nr 211, Nr 214, Nr 225) were isolated through screening of the axr1-12 by spraying of 0.01 mM 2,4-D or 0.1 mM GA$_3$. However, most of these mutants could also develop siliques in next generations without 2,4-D or GA$_3$ treatment.

Cytokinins were thought to be involved in the phase II of fruit development during which the highest cell division activity in pericarp and placental tissue occurs (Bohner and Bangerth, 1988). In this stage, a high cytokinin level was found in developing seeds (Bohner and Bangerth, 1988). It is most probably generated by transport from outside tissues to the developing seeds (Bohner and Bangerth, 1988). It was thought that an outward flow of cytokinin from the developing seed regulated cell division activity (Gillapsy, *et al.*, 1993). The presented data showed that the application of BAP on siliques could not induce the silique growth of *axr1-12* and only gave a very weak stimulation to that of Col (Figure II-1, Figure II-2), suggesting that the exogenous cytokinin cannot efficiently influence the silique development. Maybe the function of cytokinin requires the combination with some other factors to form a positive regulator to lead the pericarp cell division. And those factors may come from the developing seed, but possibly, they are not gibberellins and auxin, since no additive effect was observed when the 2-4-D or GA$_3$ was combined with BAP to treat the siliques of *axr1-12* (Figure II-4).

Ethylene is a major regulator of the fruit ripening process. Ethylene regulates various aspects of post-pollination development with auxin, especially the coordinated development of the female and male gametophytes (Zhang and O'Neill, 1993). Some of the effects of auxin on fruiting may be mediated through the promotion of ethylene synthesis by inducing the ACC synthetase (Kende and Zeevaart, 1997; Taiz and Zeiger, 1998). When the siliques were treated with the ethylene precursor ACC, the silique growth of the wild type plants was induced, while no effect was found in the mutant *axr1-12*. This suggests that AXR1 functioning in silique development of *Arabidopsis* might affect the conversion of ACC to ethylene or the signaling of ethylene.

Pistils treated with GA$_3$ and 2,4-D could develop parthenocarpic siliques without seed development (Figure II-3). Thus, silique growth can be uncoupled from the normally linked process of seed development. Probably the endogenous hormone synthesis and the perception of an endogenous hormone, or exogenous application of specific PGR in a particular tissue are key factors in hormone-induced parthenocarpy.

4.2 Suppressors of *axr1-12*

The *AXR1* gene is required for a variety of auxin responses including auxin-induced cell proliferation (Lincoln and Estelle, 1991), auxin inhibition of root growth (Evans *et al.*,

1994) and auxin-induced gene expression (Timpte *et al.*, 1995; Abel *et al.*, 1995). The *axr1-12* mutant even has a defect in filaments growth and reduced pollen yield. Our results showed that the *AXR1* gene is required for the silique response to PGRs too. The mutation in *AXR1* gene (*axr1-12*) leads to siliques less sensitivity to 2,4-D, BAP and ACC (Figure II-1; Figure II-2).

To gain insight into parthenocarpic fruit development, *axr1-12* was used as the original mutant to screen secondary mutants that could develop siliques apparently and /or were more sensitive to auxin or gibberellins in silique development. 11 mutants from M1 and 31 mutants from M2 were isolated. All these 42 mutants are dramatically different from the original mutant *axr1-12* in the phenotype. Generally, they have similar phenotypes to the wild type Col. They can develop siliques; they have relatively smooth leaf edges and show apical dominance; and their root growth are more sensitive in response to 2,4-D. These obviously revealed that the new mutations suppress, at least partially, the defects the *axr1-12* phenotype.

We expected to isolate some parthenocarpic mutants and find some new gene that could control fruit development. Two parthenocarpy mutants (Nr 220, Nr 230) were isolated in the screen of *axr1-12*. These two mutants could develop siliques uncoupled from seed development**.** Unfortunately, no offspring of them was generated regardless of the effort on rescuing them, indicating they might be lethal mutants. However, it does not mean all the parthenocarpy mutants should be lethal mutants. Vivian-Smith *et al.* (2001) isolated and characterized a parthenocarpy mutant called *fwf* (fruit without fertilization) from the male sterile *pistillata* (*pi*) mutant, this *fwf* mutant had a mutation in the *FWF* gene which is involved in processes repressing the development of silique tissues in the absence of fertilization. In our study, another mutant Nr 225 could also develop siliques without fertilization when it was isolated from M2 with GA$_3$ spraying. Further study should be done to make it clear whether this mutant is a true parthenocarpic mutant sensitive to GA$_3$.

Genetic analysis of 8 mutants based on the auxin response of roots showed that 7 of them carry recessive mutation and 1 of them (Nr 214) carries a semi-dominant mutation.

Further analysis of 5 recessive mutants documented that all carry the second mutation different from the *axr1-12* locus. Allelic analysis of three mutants (Nr 23, Nr 43 and Nr 53) revealed that two of them (Nr 43 and Nr 53) carry allelic suppressor mutations, therefore at least two different suppressor loci were found among these recessive mutants. These recessive mutations, here named **supa** (suppressor of axr1-12), have no specific phenotype in *AXR1* wild type background. They exhibit their phenotype only when the plants carry the original mutation *axr1-12* (Table II-5). It indicates that the defect of *axr1-12* in auxin signal transduction pathway can be restored by the mutation

in *SUPA* genes. These SUPAs likely function in the same pathway as AXR1 and act downstream of AXR1. The *AXR1* gene is reported to have important role in regulating protein degradation. It encodes an enzyme subunit, which forms a heterodimeric enzyme with another subunit ECR1, to be responsible for the activation of the ubiquitin-related protein RUB (Leyser *et al.*, 1993; del Pozo *et al.*, 2002). The activated RUB is further required to modify the AtCUL1, a component of the SCF ubiquitin ligase, and to activate SCF (Gray *et al.*, 1999; 2000; 2003; Figure II-30). Thus, SUPAs are speculated to act as repressors in the ubiquitin pathway. The degradation of SUPAs might lead to the release of early auxin response genes and might activate the auxin response. The mutation in the *SUPA* genes could result in the loss of function (Figure II-30). Or the SUPA might act as a target of a repressor, and the mutation in the *SUPA* could disable the interaction between the repressor and *SUPA* without influence on the function of *SUPA* in the auxin response.

Cernac *et al.* (1997) performed a screen for suppressors of *axr1-3* based on the auxin resistance in root elongation. At least three second-site suppressor loci were identified and one gene *SAR1* was characterized. It is unclear if any *supa* mutants are allelic of *sar1*.

The semi-dominant mutant Nr 214 carries a mutation separated from AXR1. This mutant has the phenotypic characters as those recessive mutants, *e.g.*, it can develop siliques, shows apical dominance and is more sensitive to 2,4-D. Additionally, it exhibited epinasty of fruit petioles when it was isolated from M2 by spraying 2,4-D. But this character did not appear in its progeny when no 2,4-D was applied. The formation of fruit petioles epinasty might be caused by the elevation of ethylene level (Taiz and Zeiger, 1998) which was induced by 2,4-D (Woeste et al., 1999). The mutation in Nr 214 can be expressed in *AXR1* background after one times backcross to wild type Col. The *AXR1* wild type background plant carrying the mutation was more sensitive to 2,4-D than wild type Col. In F2 generation, the phenotype displayed the Mendelian segregation of co-dominant trait (Table II-4). However, the suppressor trait was not stably inherited in next generations of selfing (Figure II-27). Furthermore, the seed germination potential decreased. Hence, it caused much uncertainty for the gene mapping because gene mapping is strictly based on the visible segregated phenotype (Glick and Thompson, 1993).

4.3 Gravitropism of suppressor lines

The gravitropism in some auxin resistant mutants in *Arabidopsis* (*e.g. axr1* and *axr4*) is partly inhibited (Yamamoto and Yamamoto, 1999), whereas the gravitropism in other mutants (*e.g. axr2*, *axr3*, *aux1* and *agr1*) is severely impaired (Taiz and Zeiger, 1998;
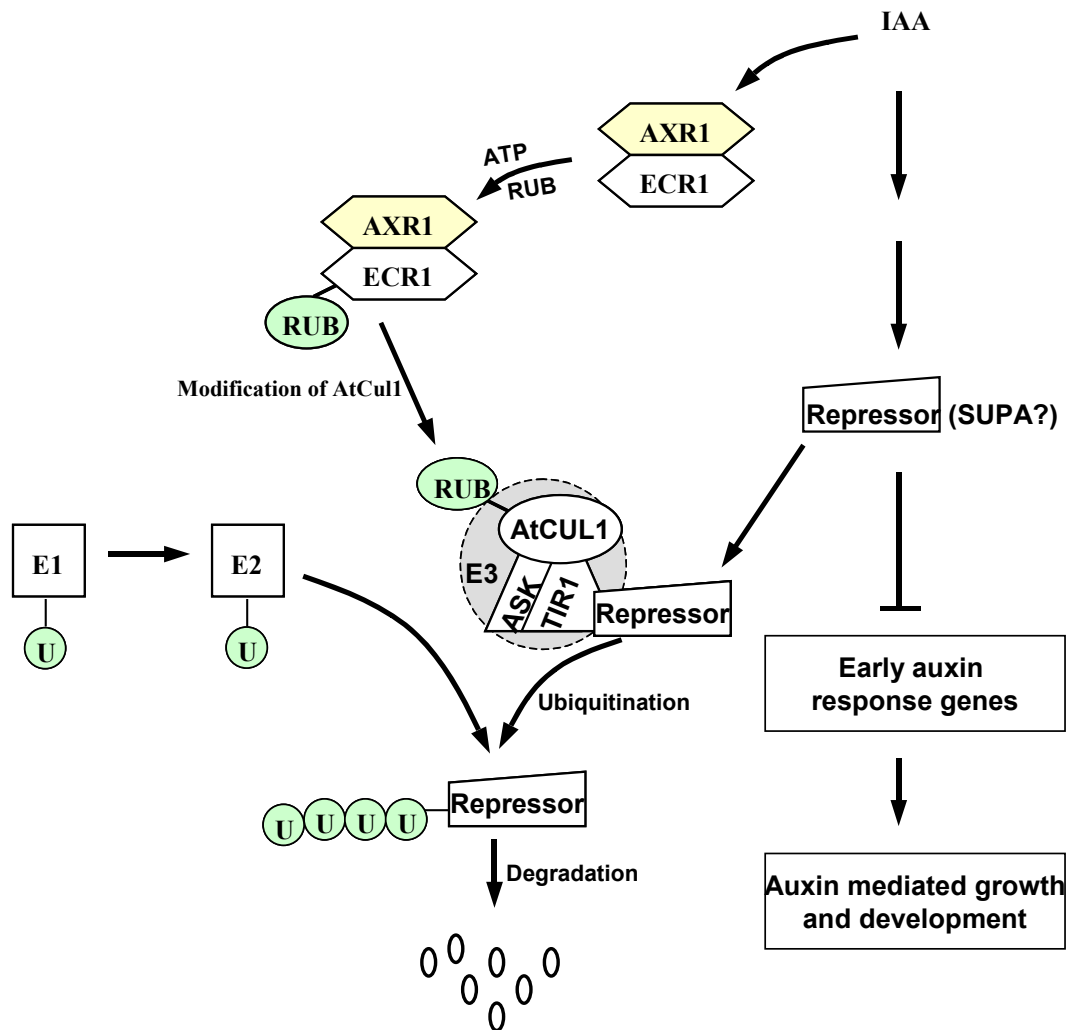
Figure II-30. Hypothetical model for auxin response mediated by the ubiquitination pathway. Step1: IAA induces the heterodimarization of AXR1 and ECR1. The AXR1/ECR1 binds RUB protein to modify AtCUL1. The SCF ubiquitin ligase is then activated and ubiquitinates the repressor. The degradation of the repressor derepresses the response pathway resulting in the activation of the early auxin response genes. E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, SCF (complex of Skp1, Cdc53 and F-box proteins) ubiquitin ligase; RUB, ubiquitin-related protein. (According to Taiz and Zeiger, 1998; Gray *et al.*, 1999).

Marchant *et al.*, 1999; Chen *et al.*, 1998). In our experiment the dark-grown etiolated *axr1-12* seedlings also revealed partial loss of gravitropism (Figure II-19). These findings suggest that auxin signaling is involved in the gravitropism. According to the Cholodny-Went model, auxin-promoted tropism is regulated by polar transport of auxin.

Auxin is transported laterally to the lower side during gravitropism and stimulate the root growth of other side. Thus, AXR1 - AXR4, AUX1 and AGR1 are likely related to auxin polar transport.

The secondary mutants (Nr 23, Nr 211, Nr 214 and Nr 225) of *axr1-12* had different phenotypes concerning root gravitropism. Nr 23, Nr 211 and Nr 214 did not restore gravitropism of *axr1-12*. The Nr 214 was impaired the gravitropism even more severe than *axr1-12*. The Nr 225 suppressor restored it slightly but still could not match the response of wild type Col (Figure II-19). The different behaviour in root gravitropism of these mutants suggests that the mutated genes are probably involved in polar auxin transport or have different function during intra- and inter-cellular signaling processes.

Light is known to influence root gravitropism (Lu and Feldman, 1997). The partial gravitropism displayed on the dark-grown *axr1-12* and the suppressors was not observed in light-grown seedlings, implying the cross-interaction of auxin and light signalling.

4.4 Apical hook formation in suppressor lines

The apical hook of hypocotyls in dark-grown seedlings is an important physiological phenomenon of dicotyledonous plants. The plant hormones ethylene and auxin have been implicated to regulate the differential growth in this process. Auxin functions as a stimulator in the cell expansion and hypocotyls elongation while ethylene has an antagonistic effect on it. Exogenous ethylene treatment could increase the bending of the hypocotyls of some etiolated seedlings (Lehman at el., 1996). Precise regulation of both the level and the distribution of auxin, which is produced in the cotyledons and transported in a polar manner from the apex (Lomax *et al.*, 1995), may be required for the establishment of a hormone gradient and for differential elongation of cells in the hypocotyls. Etiolated seedlings treated with the polar auxin transport inhibitors naphthylphthalamic acid (NPA) and triiodobuteric acid (TIBA) were hookless, the hookless mutant *hls3*, in which the apical hook does not form, produced 6-fold higher levels of endogenous free IAA (Lehman *et al.*, 1996), indicating an essential role for auxin in the establishment of differential elongation of cells required for hypocotyls curvature in *Arabidopsis*.

Auxin resistant mutants show much reduced differential growth in the apical hook (Schwark and Schierle, 1992). Lehman *et al.* (1996) and Cernac *et al.* (1997) reported that dark-grown *axr1-12* seedlings are unable to form an apical hook. In this aspect Nr 211 is similar to *axr1-12*. The other suppressors, Nr 23, Nr 214 and Nr 225, are able to recover the defect in *axr1-12* (Figure II-19). It's not clear whether different phenotypes in apical hook formation of these mutants are due to the different ability of auxin polar transport or only due to the different sensitivity of perceived auxin.

4.5 Seed development in suppressor lines

Seed and seed-like structures can develop in self-crossed plants of the group B mutants (Figure II-8), implying that the second mutations might be able to lead to the embryo death and the corresponding genes might play a role in apomictic seed development. These mutants have a similar phenotype as the *mea* (Grossiniklaus *et al.*, 1998), *fis* (Chaudhury *et al.* 1997, Luo *et al.*, 1999) and *fie* (Ohad *et al.*, 1996) mutants. In those mutants seed development is initiated in the absence of fertilization, the endosperm is formed, the seed coat is differentiated, while no functional embryo is developed. Data suggest that the auxin signaling is likely involved in apomictic seed development event.

In summary, several suppressors were screened from *axr1-12*. At least three distinct mutations (*e.g.* the mutations in Nr 23, Nr 43, Nr 214) were identified. All the suppressors restored the phenotype of *axr1-12* concerning silique development, auxin sensitivity and leaf morphology. Most of them restored the apical dominance. However, these mutants have their own behaviours such as in root gravitropism, apical hook formation. It suggests that the identified mutations act in different steps of auxin signal transduction.

Appendix

Appendix 1. Media recipes

Medium for yeast culture

YPD medium (/L):

Difco peptone	20 g
Yeast extract	10 g
Agar	20 g (for plates only), pH 5.8

Allow medium to cool to about 55 ^{o}C and add glucose to 2%.

SD medium (/ L):

This is a minimal medium used in yeast transformations to select and test for specific phenotypes.

Yeast nitrogen base without amino acids	6.7 g
10x DO (dropout solution)	100 ml
Agar	20 g (for plates only)

Add 850ml H$_2$O and adjust pH to 5.8, and autoclave. Allow medium to cool to about 55°C and add glucose to 2%. For screening, 8 mM 3-AT was added to the selective medium to inhibit low level of His3 expression.

10XDO (mg/L):

L-Isoleucine	300	L-Methionine	200
L-Valine	1500	L-Phenylalanine	500
L-Adenine hemisulfate salt	200	L-Threonine	2000
L.Arginine HCl	200	L-Tryptophan	200
L-Histidine HCl monohydrate	200	L-Tyrosine	300
L-Leucine	1000	L-Uracil	200
L-Lysine HCl	300		

Medium for bacteria culture

LB medium (/ L):

Bacto Tryptone	10.0 g	NaCl	10.0 g
Yeast extract	5.0 g	Agar	18 g (for plates only)

SOC: This rich medium is used in transformation.

Bacto tryptone	2%	MgCl$_2$	10 mM
Bacto yeast extract	0.5%	MgSO$_4$	10 mM
NaCl	10 mM	Glucose	20 mM
KCl	2.5 mM		

M9 agar medium (/ L): This medium is used for HB101 transformed of the yeast

plasmids.

5xM9 salts	200 ml	1 M Thiamine	1 ml
1M MgSO$_4$	2 ml	10mg/ml Proline	4 ml
1M CaCl$_2$	0.1 ml	10x DO	100 ml
40% Glucose	10 ml	100mg/ml Ampcillin	500 µl
Agarose	18 g		

5x M9 salts (/L):

Na$_2$HPO$_4$.7H$_2$O	64 g	NaCl	2.5 g
KH$_2$PO$_4$	14 g	NH$_4$Cl	5 g

If necessary, the medium can be supplemented with appropriate antibiotics.
Addition of antibiotics:
Ampicillin: 50 µg/ ml   stock solution 100 mg/ ml in H$_2$O
Kanamycin: 50 µg/ ml   stock solution 50 mg/ ml in H$_2$O

Medium for *Agrobacterium* culture

YM-Medium(1L):

Yeast extract	0.4 g	1M K$_2$HPO$_4$	2.7 ml
Mannitol	10 g	1M KH$_2$PO$_4$	1.7 ml
5M NaCl	0.33 ml	Agar (for plates)	15 g
1M MgSO$_4$	1.6 ml	pH 7.0	

YEB Medium(1L):

Beef extract	5 g	sucrose	5 g
Yeast extract	1 g	Agar (for plates)	15 g
Peptone/Tryptone	5 g	pH 7.2 (adjusted by 1M NaOH)	

Modified MS Medium for *Arabidopsis* suspension cell culture (/ L)

MS basic micro– and macro-salts (Sigma), with 4ml of 100XB5 Vitamine and 30 g sucrose, 1 µg/ ml sterilized 2,4-D, adjust pH to 5.7, autoclaved.

100XB5 Vitamine(/ ml):

Nicotinic acid	1 mg	Pyriodixin HCl	1 mg
Thiamine HCl	10 mg	Myo-Inositol	100 mg

Appendix 2. Primers

Primers for yeast activation domain vectors

Ad f (forward):   TACCACTACAATGGATG
Ad r (reverse):   GTTGAAGTGAACTTGCG

Primers for *Arabidopsis* Glutamyl-tRNA synthetase gene

GTS f: TGGCCCGGGGGATGGATGGGATGAAGC

GTS BamH f: CGAATTCCCGGATCCTATGGATGGGATGAAGCTTTC

GTS Nco f: AACCATGGGTCGACAGATGGATGGGATGAAGCTTTC

GTS217 f: CGAATTCCCGGATCCCCGGTTTGCTCCAGAGCCAAG

GTS232 f: CGAATTCCCGGATCCTAAGGCTGCGTTGCTGAACAAG

GTS129 f: GAATTCGCGGCCGCTTCCTGATTTCTATGGC

GTS449 f: TCGGATCCTCTCAGTAAGCGCAAGCTTC

GTS129 r: GAATTCGCGGCCGCTTCCGGCAAGTCC

GTS214 r: CGGGATCCTGCAGAACCTTTCCAATCTCCGC

GTS216 r: TTGCAGGTCGACGAGTTTAACCTTTCCAATC

GTS261 r: ATAAGATCTGCTTTCTTTAGCAGGGTTAG

GTS844 r: CGGGATCCTGCAGCTTTCTTTAGCAGGGTTAG

GTS445 r: TTGCAGGTCGACAGAAGCTTGCGCTTACTGAG

GTS r: TCACTTAGCAGATCTTCCATCTGG

GTS SalI r: TTGCAGGTCGACCTCTTCCATCTGG

For *AtHB6* gene

HB6 f: CGGGATCCTAATGATGAAGAGATTAAGTAG

HB6NotI f: ATAAGAATGCGGCCGCGATGATGAAGAGATTAAGTAG

HB6dC269.BamH r: AAGGCCTGGATCCGAAATTACCTCCTGGAACC

HB6dC269.PstI r: AAGGCCTCTGCAGAAATTACCTCCTGGAACC

HB6 r: CGGGATCCTGCAGTCAATTCCAATGATCAACG

For *ABI1* gene

ABI1 Not f: ATAAGAATGCGGCCGCAATGGAGGAAGTATCTCC

ABI1 1736 r: AAGGCCTGGATCCTCAGTTCAAGGGTTTGCTC

For *ABI2* gene

ABI2 Notf: ATAAGAATGCGGCCGCATGGACGAAGTTTCTCCTGC

ABI2 Notr: ATAAGATCAGCGGCCGCTCAATTCAAGGATTTGCTC

For *AtHB7* gene

HB7f: CGGGATCCTAATGGAGGTTCTGATTCC

HB7.10f: ACCGGAATTCATGGAGGTTCTGATTCC

HB7.NotIf: ATAAGAATGCGGCCGCCATGGAGGTTCTGATTCC

HB7 420r: AAGGCCTAGATCTCGTCGCCTCTTTTAGCC

HB7.r: CGGGATCCTGCAGTCATGACCAAAAATCCCG

Appendix 3. cDNA and amino acid sequences of the candidates identified by AtHB6 using yeast two-hybrid system

1. cDNA sequences of the candidates identified by AtHB6 using yeast two-hybrid system

1.1 Comparison of the nucleotide sequence of the insert of candidate in class A to *Arabidopsis GluRS***. All alignment was done using the MultAlin (Corpet F., 1988).**

```
              1        10        20        30        40        50        60        70        80        90
              |---------+---------+---------+---------+---------+---------+---------+---------+---------|
       GluRS  CGTCTCTCTCTCAAACCCACATTGCACAATCCATTTCTCGTTTCTCTGATTTAGATCCAAAGATGGATGGGATGAAGCTTTCGTTCCCAC
  CandidateA: CGTCTCTCTCTCAAACCCACATTGCACAATCCATTTCTCGTTTCTCTGATTTAGATCCAAAGATGGATGGGATGAAGCTTTCGTTCCCAC
   Consensus  CGTCTCTCTCTCAAACCCACATTGCACAATCCATTTCTCGTTTCTCTGATTTAGATCCAAAGATGGATGGGATGAAGCTTTCGTTCCCAC

              91       100       110       120       130       140       150       160       170       180
              |---------+---------+---------+---------+---------+---------+---------+---------+---------|
       GluRS  CGGAAAGTCCACCACTTTCAGTCATCGTTGCTCTTTCTCTCTCAGCTTCTCCGGTGACGATTGATTCTTCCGCCGCTGCAACAACCGTCC
  CandidateA: CGGAAAGTCCACCACTTTCAGTCATCGTTGCTCTTTCTCTCTCAGCTTCTCCGGTGACGATTGATTCTTCCGCCGCTGCAACAACCGTCC
   Consensus  CGGAAAGTCCACCACTTTCAGTCATCGTTGCTCTTTCTCTCTCAGCTTCTCCGGTGACGATTGATTCTTCCGCCGCTGCAACAACCGTCC

              181      190       200       210       220       230       240       250       260       270
              |---------+---------+---------+---------+---------+---------+---------+---------+---------|
       GluRS  CTTCTTTTGTCTTCTCCGACGGGAGGAAATTGAATGGAGCCACCGTTCTTCTTCGCTATGTTGGTCGATCAGCGAAAAAGCTTCCTGATT
  CandidateA: CTTCTTTTGTCTTCTCCGACGGGAGGAAATTGAATGGAGCCACCGTTCTTCTTCGCTATGTTGGTCGATCAGCGAAAAAGCTTCCTGATT
   Consensus  CTTCTTTTGTCTTCTCCGACGGGAGGAAATTGAATGGAGCCACCGTTCTTCTTCGCTATGTTGGTCGATCAGCGAAAAAGCTTCCTGATT

              271      280       290       300       310       320       330       340       350       360
              |---------+---------+---------+---------+---------+---------+---------+---------+---------|
       GluRS  TCTATGGCAACAATGCTTTTGATTCTTCTCAGATTGATGAGTGGGTAGATTACGCATCTGTCTTCTCTTCTGGTTCAGAGTTTGAGAATG
  CandidateA: TCTATGGCAACAATGCTTTTGATTCTTCTCAGATTGATGAGTGGGTAGATTACGCATCTGTCTTCTCTTCTGGTTCAGAGTTTGAGAATG
   Consensus  TCTATGGCAACAATGCTTTTGATTCTTCTCAGATTGATGAGTGGGTAGATTACGCATCTGTCTTCTCTTCTGGTTCAGAGTTTGAGAATG

              361      370       380       390       400       410       420       430       440       450
              |---------+---------+---------+---------+---------+---------+---------+---------+---------|
       GluRS  CTTGTGGTCGTGTTGATAAGTATCTCGAGAGTAGCACGTTTCTTGTTGGCCATTCTCTTTCCATTGCTGATGTCGCTATTTGGTCAGCTC
  CandidateA: CTTGTGGTCGTGTTGATAAGTATCTCGAGAGTAGCACGTTTCTTGTTGGCCATTCTCTTTCCATTGCTGATGTCGCTATTTGGTCAGCTC
   Consensus  CTTGTGGTCGTGTTGATAAGTATCTCGAGAGTAGCACGTTTCTTGTTGGCCATTCTCTTTCCATTGCTGATGTCGCTATTTGGTCAGCTC

              451      460       470       480       490       500       510       520       530       540
              |---------+---------+---------+---------+---------+---------+---------+---------+---------|
       GluRS  TTGCTGGAACTGGTCAAAGATGGGAAAGTTTGAGGAAATCTAAAAAGTATCAGAGTCTTGTTAGATGGTTCAATTCGATATTAGACGAGT
  CandidateA: TTGCTGGAACTGGTCAAAGATGGGAAAGTTTGAGGAAATCTAAAAAGTATCAGAGTCTTGTTAGATGGTTCAATTCGATATTAGACGAGT
   Consensus  TTGCTGGAACTGGTCAAAGATGGGAAAGTTTGAGGAAATCTAAAAAGTATCAGAGTCTTGTTAGATGGTTCAATTCGATATTAGACGAGT

              541      550       560       570       580       590       600       610       620       630
              |---------+---------+---------+---------+---------+---------+---------+---------+---------|
       GluRS  ACAGTGAGGTGCTTAACAAGGTTCTAGCAACTTATGTTAAGAAAGGATCAGGGAAGCCTGTTGCTGCACCTAAGTCTAAAGATAGCCAAC
  CandidateA: ACAGTGAGGTGCTTAACAAGGTTCTAGCAACTTATGTTAAGAAAGGATCAGGGAAGCCTGTTGCTGCACCTAAGTCTAAAGATAGCCAAC
   Consensus  ACAGTGAGGTGCTTAACAAGGTTCTAGCAACTTATGTTAAGAAAGGATCAGGGAAGCCTGTTGCTGCACCTAAGTCTAAAGATAGCCAAC

              631      640       650       660       670       680       690       700       710       720
              |---------+---------+---------+---------+---------+---------+---------+---------+---------|
       GluRS  AAGCTGTGAAAGGAGATGGTCAGGATAAAGGTAAGCCTGAAGTGGACTTGCCGGAAGCGGAGATTGGAAAGGTTAAACTCCGGTTTGCTC
  CandidateA: AAGCTGTGAAAGGAGATGGTCAGGATAAAGGTAAGCCTGAAGTGGACTTGCCGGAAGCGGAGATTGGAAAGGTTAAACTCCGGTTTGCTC
   Consensus  AAGCTGTGAAAGGAGATGGTCAGGATAAAGGTAAGCCTGAAGTGGACTTGCCGGAAGCGGAGATTGGAAAGGTTAAACTCCGGTTTGCTC
```

```
            721   730    740    750    760    770    780    790    800    810
            |---------+----------+----------+----------+----------+----------+----------+----------+----------+----------|
    GluRS   CAGAGCCAAGTGGTTATCTTCACATAGGACATGCTAAGGCTGCGTTGCTGAACAAGTATTTCGCTGAGCGTTACCAAGGGGAAGTGATTG
CandidateA: CAGAGCCAAGTGGTTATCTTCACATAGGACATGCTAAGGCTGCGTTGCTGAACAAGTATTTCGCTGAGCGTTACCAAGGGGAAGTGATTG
Consensus   CAGAGCCAAGTGGTTATCTTCACATAGGACATGCTAAGGCTGCGTTGCTGAACAAGTATTTCGCTGAGCGTTACCAAGGGGAAGTGATTG

            811   820    830    840    850    860    870    880    890    900
            |---------+----------+----------+----------+----------+----------+----------+----------+----------+----------|
    GluRS   TGCGTTTTGATGATACTAACCCTGCTAAAGAAAGCAATGAGTTTGTGGATAATCTTGTGAAGGATATTGGGACCTTGGGGATCAAGTATG
CandidateA: TGCGTTTTGATGATACTAACCCTGCTAAAGAAAGC
Consensus   TGCGTTTTGATGATACTAACCCTGCTAAAGAAAGC...........................................................

            901   910    920    930    940    950    960    970    980    990
            |---------+----------+----------+----------+----------+----------+----------+----------+----------+----------|
    GluRS   AGAAAGTGACATACACTTCGGACTATTTTCCTGAATTGATGGATATGGCGGAAAAACTGATGCGTGAGGGTAAGGCATATGTTGATGACA
CandidateA:
Consensus   ...........................................................................................

            2251  2260   2270   2280   2290   2300   2310   2321
            |---------+----------+----------+----------+----------+----------+----------+|
    GluRS   TTATGAACATTGTGTTGAGAAATTTGCTCTTTAGGTTTTAAAACAATGAAAACAAAGCTTTTTATCAGGCG
CandidateA:
Consensus   ...............................................................
```

210

1.2 Comparison of the nucleotide sequence of the insert of candidate in class B to *Arabidopsis AtHB7*.

```
             1        10        20        30        40        50        60        70        80        90
             |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
ATHB7                        GAGAAGTTCATGGAGGTTCTGATTCCGGT---CGATGAGATGACAGAAGGTGGAGAATATTCTCCGGCGATGATGTCAG
CandidateB   AAAATAAAAATAAAGAAAACATAAATCCTCTGAGGAAAAATTCCGATGAGATGACAGAAGGTGGAGAATATTCTCCGGCGATGATGTCAG
Consensus    ............aAaaAaaaCATaaAgccTCTGAggaaaaa...CGATGAGATGACAGAAGGTGGAGAATATTCTCCGGCGATGATGTCAG

             91       100       110       120       130       140       150       160       170       180
             |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
ATHB7        CAGAGCCATTCTTGACCATGAAGAAGATGAAGAAGAGCAACCACAACAAGAACAATCAGAGAAGGTTTAGCGACGAGCAGATCAAGTCAC
CandidateB   CAGAGCCATTCTTGACCATGAAGAAGATGAAGAAGAGCAACCACAACAAGAACAATCAGAGAAGGTTTAGCGACGAGCAGATCAAGTCAC
Consensus    CAGAGCCATTCTTGACCATGAAGAAGATGAAGAAGAGCAACCACAACAAGAACAATCAGAGAAGGTTTAGCGACGAGCAGATCAAGTCAC

             181      190       200       210       220       230       240       250       260       270
             |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
ATHB7        TGGAGATGATGTTTGAGTCTGAGACAAGGCTTGAGCCAAGGAAGAAGGTTCAATTAGCTAGAGAGCTAGGGTTGCAGCCGAGGCAAGTGG
CandidateB   TGGAGATGATGTTTGAGTCTGAGACAAGGCTTGAGCCAAGGAAGAAGGTTCAATTAGCTAGAGAGCTAGGGTTGCAGCCGAGGCAAGTGG
Consensus    TGGAGATGATGTTTGAGTCTGAGACAAGGCTTGAGCCAAGGAAGAAGGTTCAATTAGCTAGAGAGCTAGGGTTGCAGCCGAGGCAAGTGG

             271      280       290       300       310       320       330       340       350       360
             |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
ATHB7        CTATATGGTTTCAGAACAAGAGGGCTCGTTGGAAATCCAAGCAGCTCGAGACTGAGTACAACATTCTCAGACAAAACTACGACAACTTGG
CandidateB   CTATATGGTTTCAGAACAAGAGGGCTCGTTGGAAATCCAAGCAGCTCGAGACTGAGTACAACATTCTCAGACAAAACTACGACAACTTGG
Consensus    CTATATGGTTTCAGAACAAGAGGGCTCGTTGGAAATCCAAGCAGCTCGAGACTGAGTACAACATTCTCAGACAAAACTACGACAACTTGG

             361      370       380       390       400       410       420       430       440       450
             |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
ATHB7        CTTCTCAGTTCGAGTCCTTAAAGAAAGAAAAACAAGCTTTAGTCTCTGAGTTGCAGAGGCTAAAAGAGGCGACG-CAAAAGAAGACACAG
CandidateB   CTTCTCAGTTCGAGTCCTTAAAGAAAGAAAAACAAGCTTTAGTCTCTGAGTTGCAGAGGCTAAAAGAGGCGACGGCCTTCGTGGCCTCGA
Consensus    CTTCTCAGTTCGAGTCCTTAAAGAAAGAAAAACAAGCTTTAGTCTCTGAGTTGCAGAGGCTAAAAGAGGCGACG.CaaaaGaaGaCaCaa

             451      460       470       480       490       500       510       520       530       540
             |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
ATHB7        GAGGAGGAAAGGCAGTGTAGTGGAGATCAAGCGGTGGTTGCTCTAAGCAGCACACATCATGAATCAGAAAACGAAGAGAACCGGAGGCGT
CandidateB   GAGAT-------CTATGAATCGTAGATA
Consensus    GAGaa.......CaaTGaAgcGgAGATa..........................................................

             541      550       560       570       580       590       600       610       620       630
             |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
ATHB7        AAACCGGAAGAGGTTAGACCGGAGATGGAGATGAAAGATGATAGGGGTCATCATGGGGTTATGTGTGATCATCATGATTATGAAGATGAT
CandidateB
Consensus    ........................................................................................

             1081     1090      1100      1110      1120      1130      1140      1150      1160      1170
             |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
ATHB7        TAAGTAGTAAGTACTCGTCATGCTTTGTAGCTAAGTAGTAAGTTTAAGACTAAATTTGGATGAATAAAAATCAAAAGCAGGAGAAATCAT
CandidateB
Consensus    ........................................................................................

             1171  11801184
             |---------+---|
ATHB7        AAAAAAAAAAAAAA
CandidateB
Consensus    ..............
```

1.3 Comparison of the insert nucleotide sequence of the candidate AV-72 in class C to *Arabidopsis 18s rRNA* gene.

```
           1171 1180      1190      1200      1210      1220      1230      1240      1250      1260
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
     18s   GAGAAATCAAAGTTTTTGGGTTCCGGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAG
   AV-72
Consensus  .........................................................................................

           1261 1270      1280      1290      1300      1310      1320      1330      1340      1350
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
     18s   CCTGCGGC-TTAATTTGACTCAACACGGGGAAACTTACCAGGTCCAGACATAGTAAGGATTGACAGACTGAGAGCTCTTTCTTGATTCTA
   AV-72    TTGCGGGGTTTTTCAGTATCTAC-----GATTCATA----GATCTCTCGAGGCCACGAAGGCC---CTGAGAGCTCTTTCTTGATTCTA
Consensus  .cTGCGGc.TTaaTcaGaaTCaAC.....GAaaCaTA....GacCacaCaaaGcaAcGAagGaC...CTGAGAGCTCTTTCTTGATTCTA

           1351 1360      1370      1380      1390      1400      1410      1420      1430      1440
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
     18s   TGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTCAGCCTGCTAACTAGCT
   AV-72   TGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTCAGCCTGCTAACTAGCT
Consensus  TGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTCAGCCTGCTAACTAGCT

           1441 1450      1460      1470      1480      1490      1500      1510      1520      1530
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
     18s   ACGTGGAGGCATCCCTTCACGGCCGGCTTCTTAGAGGGACTATGGCCGTTTAGGCCAAGGAAGTTTGAGGCAATAACAGGTCTGTGATGC
   AV-72   ACGTGGAGGCATCCCTTCACGGCCGGCTTCTTAGAGGGACTATGGCCGTTTAGGCCAAGGAAGTTTGAGGCAATAACAGGTCTGTGATGC
Consensus  ACGTGGAGGCATCCCTTCACGGCCGGCTTCTTAGAGGGACTATGGCCGTTTAGGCCAAGGAAGTTTGAGGCAATAACAGGTCTGTGATGC

           1531 1540      1550      1560      1570      1580      1590      1600      1610      1620
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
     18s   CCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGATGTATTCAACGAGTTCACACCTTG-CCGACAGGCCCGGGTAATCTTTGAAATTT
   AV-72   CCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGATGTATTCAACGAGTTCACACCTTGGCCGACAGGCCCGGGTAATCTTTGAAATTT
Consensus  CCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGATGTATTCAACGAGTTCACACCTTG.CCGACAGGCCCGGGTAATCTTTGAAATTT

           1621 1630      1640      1650      1660      1670      1680      1690      1700      1710
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
     18s   CATCGTGATGGGGATAGATCATTGCAATTGTTGGTCTTCAACGAGGAATTCCTAGTAAGCGCGAGTCATCAGCTCGCGTTGACTACGTCC
   AV-72   CATCGTGATGGGGATAGATCATTGCAATTGTTGGTCTTCAACAAG
Consensus  CATCGTGATGGGGATAGATCATTGCAATTGTTGGTCTTCAACaAG.............................................

           1711 1720      1730      1740      1750      1760      1770      1780      1790      1800
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
     18s   CTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATTGAATGATCCGGTGAAGTGTTCGGATCGCGGCGACGTGGGTGGTTCGCCGCCC
   AV-72
Consensus  .........................................................................................
```

1.4 Comparison of the nucleotide sequence of candidate in class D to *Arabidopsis* unknown protein gene *At3g04830*.

```
            1         10        20        30        40        50        60        70        80        90
            |---------+---------+---------+---------+---------+---------+---------+---------+---------|
At3g04830                                          ACAAATTTCATAAGGTTCAGAGCAAATCGGAGAGAGAGAGATGGTGACGAA
       D1   TCTCGAGGATCCGAATTCGCGGCCGCGTCGACCAGATCCACAAATTTCATAAGGTTCAGAGCAAATCGGAGAGAGAGAGATGGTGACGAA
Consensus   ...........................................ACAAATTTCATAAGGTTCAGAGCAAATCGGAGAGAGAGAGATGGTGACGAA

            91        100       110       120       130       140       150       160       170       180
            |---------+---------+---------+---------+---------+---------+---------+---------+---------|
At3g04830   AACGGAGGAGATACAGTTAAACCAACTCGAGAATCAAGTTGAGAATGGAGGAGGAGGTGTTTGGGAGTATCTGTGCCTCGTTCGTAAGCT
       D1   AACGGAGGAGATACAGTTAAACCAACTCGAGAATCAAGTTGAGAATGGAGGAGGAGGTGTTTGGGAGTATCTGTGCCTCGTTCGTAAGCT
Consensus   AACGGAGGAGATACAGTTAAACCAACTCGAGAATCAAGTTGAGAATGGAGGAGGAGGTGTTTGGGAGTATCTGTGCCTCGTTCGTAAGCT

            181       190       200       210       220       230       240       250       260       270
            |---------+---------+---------+---------+---------+---------+---------+---------+---------|
At3g04830   CAAGGTTCGCCGATCAGAGATTGTGCTCAAGCATGGTCTCTCGATCTTGAACGATTCGGGAAAGCGATCCGCTCTTGGTCCAGATGAATG
       D1   CAAGGTTCGCCGATCAGAGATTGTGCTCACGCATGGTCTCTCGATCTTGAACGATTCGGGAAAGCGATCCGCTCTTGG
Consensus   CAAGGTTCGCCGATCAGAGATTGTGCTCAaGCATGGTCTCTCGATCTTGAACGATTCGGGAAAGCGATCCGCTCTTGG............

            271       280       290       300       310       320       330       340       350       360
            |---------+---------+---------+---------+---------+---------+---------+---------+---------|
At3g04830   GACCCTGTATGAGCAGGTAGCAATTGCAGCTATGGACTGTCAATCTCTCGGTGTTGCACAGGTTCTAAAGAAGAAATTTCCGGAGAGCAA
       D1
Consensus   .........................................................................................



            1081 1090       1100      1110      1120      1130      1140      1150      1160      1170
            |---------+---------+---------+---------+---------+---------+---------+---------+---------|
At3g04830   TTTTTTCTTATTCACTTGGCTATATTGTTGAATCACTGATACGGTTTACTAAGGTGTGTTTAATCAAATAATAAATTAAACTAATCCAAA
       D1
Consensus   .........................................................................................

            1171 1180 1183
            |---------+--|
At3g04830   AAAAAAAAAAAAA
       D1
Consensus   ............
```

2. Amino acid sequences of candidates identified by AtHB6 using yeast two-hybrid system

2.1 Comparison of the amino acid sequences of the candidate in class A to *Arabidopsis* GluRS.

```
          1         10        20        30        40        50        60        70        80        90
          |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
     GluRS                    MDGMKLSFPPESPPLSVIVALSLSASPVTIDSSAAATTVPSFVFSDGRKLNGATVLLRYYGRSAKKLPDF
CandidateA SLSQTHIAQSISRFSDLDPKMDGMKLSFPPESPPLSVIVALSLSASPVTIDSSAAATTVPSFVFSDGRKLNGATVLLRYVGRSAKKLPDF
 Consensus ....................MDGMKLSFPPESPPLSVIVALSLSASPVTIDSSAAATTVPSFVFSDGRKLNGATVLLRYVGRSAKKLPDF

          91        100       110       120       130       140       150       160       170       180
          |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
     GluRS YGNNAFDSSQIDEWVDYASVFSSGSEFENACGRVDKYLESSTFLVGHSLSIADVAIWSALAGTGQRWESLRKSKKYQSLVRWFNSILDEY
CandidateA YGNNAFDSSQIDEWVDYASVFSSGSEFENACGRVDKYLESSTFLVGHSLSIADVAIWSALAGTGQRWESLRKSKKYQSLVRWFNSILDEY
 Consensus YGNNAFDSSQIDEWVDYASVFSSGSEFENACGRVDKYLESSTFLVGHSLSIADVAIWSALAGTGQRWESLRKSKKYQSLVRWFNSILDEY

          181       190       200       210       220       230       240       250       260       270
          |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
     GluRS SEVLNKVLATYYKKGSGKPVAAPKSKDSQQAVKGDGQDKGKPEVDLPEAEIGKVKLRFAPEPSGYLHIGHAKAALLNKYFAERYQGEVIV
CandidateA SEVLNKVLATYYKKGSGKPVAAPKSKDSQQAVKGDGQDKGKPEVDLPEAEIGKVKLRFAPEPSGYLHIGHAKAALLNKYFAERYQGEVIV
 Consensus SEVLNKVLATYYKKGSGKPVAAPKSKDSQQAVKGDGQDKGKPEVDLPEAEIGKVKLRFAPEPSGYLHIGHAKAALLNKYFAERYQGEVIV

          271       280       290       300       310       320       330       340       350       360
          |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
     GluRS RFDDTNPAKESNEFVDNLVKDIGTLGIKYEKVTYTSDYFPELMDMAEKLMREGKAYVDDTPREQMQKERMDGIDSKCRNHSVEENLKLWK
CandidateA RFDDTNPAKES
 Consensus RFDDTNPAKES.........................................................................

          361       370       380       390       400       410       420       430       440       450
          |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
     GluRS EMIAGSERGLQCCVRGKFNMQDPNKAMRDPVYYRCNPMSHHRIGDKYKIYPTYDFACPFVDSLEGITHALRSSEYHDRNAQYFKVLEDMG
CandidateA
 Consensus ....................................................................................

          451       460       470       480       490       500       510       520       530       540
          |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
     GluRS LRQVQLYEFSRLNLVFTLLSKRKLLWFVQTGLVDGWDDPRFPTVQGIVRRGLKIEALIQFILEQGASKNLNLMEWDKLWSINKRIIDPVC
CandidateA
 Consensus ....................................................................................

          541       550       560       570       580       590       600       610       620       630
          |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
     GluRS PRHTAVVAERRYLFTLTDGPDEPFVRMIPKHKKFEGAGEKATTFTKSIWLEEADASAISVGEEVTLMDWGNAIVKEITKDEEGRVTALSG
CandidateA
 Consensus ....................................................................................

          631       640       650       660       670       680       690       700       710       720
          |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
     GluRS VLNLQGSVKTTKLKLTWLPDTNELVNLTLTEFDYLITKKKLEDDDEVADFVNPNTKKETLALGDSNMRNLKCGDVIQLERKGYFRCDYPF
CandidateA
 Consensus ....................................................................................

          721       730       739
          |---------+---------|
     GluRS VKSSKPIVLFSIPDGRAAK
CandidateA
 Consensus ...................
```

2.2 Comparison of the amino acid sequence of the insert of candidate in class B to *Arabidopsis* AtHB7.

```
              1        10        20        30        40        50        60        70        80        90
              |---------+---------+---------+---------+---------+---------+---------+---------+---------+
     ATHB7                MTEGGEYSPAMMSAEPFLTMKKMKKSNHNKNNQRRFSDEQIKSLEMMFESETRLEPRKKVQLARELGLQPRQVA
 CandidateB   NKNKENINPLRKNSDEMTEGGEYSPAMMSAEPFLTMKKMKKSNHNKNNQRRFSDEQIKSLEMMFESETRLEPRKKVQLARELGLQPRQVA
  Consensus   ................MTEGGEYSPAMMSAEPFLTMKKMKKSNHNKNNQRRFSDEQIKSLEMMFESETRLEPRKKVQLARELGLQPRQVA

              91       100       110       120       130       140       150       160       170       180
              |---------+---------+---------+---------+---------+---------+---------+---------+---------+
     ATHB7    IWFQNKRARWKSKQLETEYNILRQNYDNLASQFESLKKEKQALVSELQRLKEATQKKTQEEERQCSGDQAYVALSSTHHESENEENRRRK
 CandidateB   IWFQNKRARWKSKQLETEYNILRQNYDNLASQFESLKKEKQALVSELQRLKEATA
  Consensus   IWFQNKRARWKSKQLETEYNILRQNYDNLASQFESLKKEKQALVSELQRLKEATa.................................

              181      190       200       210       220       230       240       250       260       270
              |---------+---------+---------+---------+---------+---------+---------+---------+---------+
     ATHB7    PEEVRPEMEMKDDKGHHGVMCDHHDYEDDDNGYSNNIKREYFGGFEEEPDHLMNIVEPADSCLTSSDDWRGFKSDTTTLLDQSSNNYPWR
 CandidateB
  Consensus   ......................................................................................

              2274
              |--|
     ATHB7     DFWS
 CandidateB
  Consensus   ....
```

2.3 Peptide encoded by candidate D.

S R G S E F A A A S T R S T N F I R F R A N R R E R D G D E N G G D T V K P T R E S S Stop

Appendix 4. cDNA and amino acid sequences of the candidates identified by AtGluRS using yeast two-hybrid system

1. cDNA sequences of the candidates identified by AtGluRS using yeast two-hybrid system

1.1 The nucleotide sequences of candidate S1

GAGCCGACATCGAAGGATCAAAAAGCAACGTCGCTATGAACGCTTGGCTGCC
ACAAGCCAGTTATCCCTGTGGTAACTTTTCTGACACCTCTAGCTTCAAATTCC
GAAGGTCTAAAGGATCGATAGGCCACGCTTTCACGGTTCGTATTCGTACTGAA
AATCAGAATCAAACGAGCTTTTACCCTTTTGTTCCACACGAGATTTCTGTTCT
CGTTGAGCTCATCTTAGGACACCTGCGTTATCTTTTAACAGATGTGCCGCCCC
AGCCAAACTCCCCACCTGACAATGTCCTCCGCCCGGATCGACCCGCCGAAGC
GAGCCTTGGGACCAAAAACAGGGGTTGTACCCCGCCTCCGGGCCTTCGTGG
CCTCGAGAGATCTATGAATCGTAGATACTGAAAAACCCCGCAGTTCCACTTCA
ACA

1.2 The nucleotide sequences of candidate S47

CCGACATCGAAGGATCAAAAAGCAACGTCGCTATGAACGCTTGGCTGCCACA
AGCCAGTTATCCCTGTGGTAACTTTTCTGACACCTCTAGCTTCAAATTCCGAA
GGTCTAAAGGATCGATAGGCCACGCTTTCACGGTTCGTATTCGTACTGAAAAT
CAGAATCAAACGAGCTTTTACCCTTTTGTTCCACACGAGATTTCTGTTCTCGT
TGAGCTCATCTTAGGACACCTGCGTTATCTTTTAACAGATGTGCCGCCCCAGC
CAAACTCCCCACCTGACAATGTCCTCCGCCCGGATCCTTCGTGGCCTCGAGA
GATCTATGAATCGTAGATACTGAAAAACCCCGCAAGTTNCACTTCAACAA

1.3 Comparison of the nucleotide sequences of candidate S8 to the *Arabidopsis thaliana* cytochrome *p450*.

```
              1        10        20        30        40        50        60        70        80        90
              |---------+---------+---------+---------+---------+---------+---------+---------+---------+
cytochrome    GTCAAACAGAAAAAAATGGATCTCTTATTGATTATAGCCGGTTTAGTAGCGGCTGCAGCCTTCTTTTTCCTCCGTAGCACGACCAAGAAA
Candidate S8
Consensus     ..........................................................................................

              91       100       110       120       130       140       150       160       170       180
              |---------+---------+---------+---------+---------+---------+---------+---------+---------+
cytochrome    TCTCTCCGGTTACCTCCGGGACCAAAAGGTCTTCCTATTATAGGAAACCTTCACCAGATGGAGAAATTCAACCCCCAACACTTCCTTTTC
Candidate S8          CTCTCGAGGCCACGAAGGCC----------------CCTTCACCAGATGGAGAAATTCAACCCCCAACACTTCCTTTTC
Consensus     ............acCTCcaGGaCaaaAAGGcC................CCTTCACCAGATGGAGAAATTCAACCCCCAACACTTCCTTTTC

              181      190       200       210       220       230       240       250       260       270
              |---------+---------+---------+---------+---------+---------+---------+---------+---------+
cytochrome    CGTCTCTCCAAGCTATACGGCCCGATTTTCACGATGAAAATCGGTGGCCGTCGCCTCGCGGTGATCTCCTCGGCCGAGCTAGCCAAGGAG
Candidate S8  CGTCTCTCCAAGCTATACGGCCCGATTTTCACGATGAAAATCGGTGGCCGTCGCCTCGCGGTGATCTCCTCGGCCGAGCTAGCCAAGGAG
Consensus     CGTCTCTCCAAGCTATACGGCCCGATTTTCACGATGAAAATCGGTGGCCGTCGCCTCGCGGTGATCTCCTCGGCCGAGCTAGCCAAGGAG

              271      280       290       300       310       320       330       340       350       360
              |---------+---------+---------+---------+---------+---------+---------+---------+---------+
cytochrome    CTACTCAAAACTCAAGACCTCAACTTCACCGCTCGTCCTCTCTTGAAAGGGCAACAAACCATGTCGTATCAAGGCCGTGAGCTTGGTTTC
Candidate S8  CTACTCAAAACTCAAGACCTCAACTTCACCGCTCGTCCTCTCTTGAAAGGGCAACAAACCATGTCGTATCAAGGCCGTGAGCTTGGTTTC
Conser        CTACTCAAAACTCAAGACCTCAACTTCACCGCTCGTCCTCTCTTGAAAGGGCAACAAACCATGTCGTATCAAGGCCGTGAGCTTGGTTTC

              361      370       380       390       400       410       420       430       440       450
              |---------+---------+---------+---------+---------+---------+---------+---------+---------+
cytochrome    GGACAGTACACCGCGTACTACCGTGAGATGAGGAAGATGTGTATGGTGAACCTCTTCAGCCCGAACCGTGTCGCAAGTTTCAGACCGGTT
Candidate S8  GGACAGTACACCGCGTACTACCGTGAGATGAGGAAGATGTGTATGGTGAACCTCTTCAGCCCGAACCGTGTCGCAAGTTTCAGACCGGTT
Consensus     GGACAGTACACCGCGTACTACCGTGAGATGAGGAAGATGTGTATGGTGAACCTCTTCAGCCCGAACCGTGTCGCAAGTTTCAGACCGGTT

              451      460       470       480       490       500       510       520       530       540
              |---------+---------+---------+---------+---------+---------+---------+---------+---------+
cytochrome    AGAGAAGAAGAGTGTCAACGGATGATGGACAAGATCTATAAAGCCGCTGATCAATCAGGCACCGTTGATCTAAGTGAGCTTCTCTTGTCT
Candidate S8  AGAGAAGAAGAGTGTCAACGGATGATGGACAAGATCTATAAAGCCGCTGATCAATCAGGCACCGTTGATCTAAGTGAGCTTCTCTTGTCT
Consens       GAGAAGAAGAGTGTCAACGGATGATGGACAAGATCTATAAAGCCGCTGATCAATCAGGCACCGTTGATCTAAGTGAGCTTCTCTTGTCT

              541      550       560       570       580       590       600       610       620       630
              |---------+---------+---------+---------+---------+---------+---------+---------+---------+
cytochrome    TTCACTAACTGTGTCGTATGTAGACAAGCTTTTGGGAAACGGTACAATGAGTACGGCACAGAGATGAAGAGATTCATAGATATCTTGTAC
Candidate S8  TTCACTAACTGTGTCGTATGTAGACAAGCTTTTGGGAAACGGTACAATGAGTACGGCACAGAGATGAAGAGATTCATAGATATCTTGTAC
Consensus     TTCACTAACTGTGTCGTATGTAGACAAGCTTTTGGGAAACGGTACAATGAGTACGGCACAGAGATGAAGAGATTCATAGATATCTTGTAC

              631      640       650       660       670       680       690       700       710       720
              |---------+---------+---------+---------+---------+---------+---------+---------+---------+
cytochrome    GAGACGCAAGCACTTTTGGGCACTCTGTTTTTTCTCCGACCTTTTCCCTTATTTCGGATTCCTTGACAACCTCACTGGTCTCAGTGCACGT
Candidate S8  GAGACGCAAGCACTTTTGGGCACTCTGTTTTTTCTCCGACCTTTTCCCTTATTTCGGATTCCTTGACAACCTCACTGGTCTCAGTGCACGT
Consensus     GAGACGCAAGCACTTTTGGGCACTCTGTTTTTTCTCCGACCTTTTCCCTTATTTCGGATTCCTTGACAACCTCACTGGTCTCAGTGCACGT

              721      730       740       750       760       770       780       790       800       810
              |---------+---------+---------+---------+---------+---------+---------+---------+---------+
cytochrome    CTCAAGAAAGCTTTCAAGGAGCTTGACACTTACCTTCAAGAACTTCTAGACGAGACTCTTGACCCTAACCGCCCCTAAACAAGAAACAGAG
Candidate S8  CTCAAGAAAGCTTTCAAGGAGCTTGACAC
Consensus     CTCAAGAAAGCTTTCAAGGAGCTTGACAC.............................................................

              811      820       830       840       850       860       870       880       890       900
              |---------+---------+---------+---------+---------+---------+---------+---------+---------+
cytochrome    AGTTTCATTGATCTTTTGATGCAGATCTACAAAGACCAACCTTTCTCCATCAAATTCACTCACGAAAATGTCAAGGCCATGATATTGGAT
Candidate S8
Consensus     ..........................................................................................

              1621 1630      1640      1650      1660  1663
              |---------+---------+---------+---------+--|
cytochrome    TTTAACGAATAAAAATGTATCAGTGCTCTTGTTTTTGGACTAG
Candidate S8
Consensus     ...........................................
```

1.4 Comparison of the nucleotide sequences of candidate S38 to an unknown *Arabidopsis thaliana* protein gene (AY045913.1).

```
         1        10        20        30        40        50        60        70        80        90
         |---------+---------+---------+---------+---------+---------+---------+---------+---------|
AY045913      ACAACTCAAAACTCTCTCCATCTCTCTTCTAAAATCAGACAAGAAAGATGGCTCAAGAGGAAGTACAGAAATCGGCTGATGTCGCTGCTG
Candidate S38                                          AGAAAGATGGCTCAAGAGGAAGTACAGAAATCGGCTGATGTCGCTGCTG
Consensus     ...........................................AGAAAGATGGCTCAAGAGGAAGTACAGAAATCGGCTGATGTCGCTGCTG

         91       100       110       120       130       140       150       160       170       180
         |---------+---------+---------+---------+---------+---------+---------+---------+---------|
AY045913      CTCCGGTGGTGAAGGAGAAACCTATTACCGATAAGGAGGTTACTATTCCTACCCCTGTGGCAGAGAAAGAGGAAGTTGCTGCTCCTGTCT
Candidate S38 CTCCGGTGGTGACGGAGAAACCTATTACCGATAAGGAGGTTACTATTCCTACCCCTGTGGCAGAGAAAGAGGAAGTTGCTGCTCCTGTCT
Consensus     CTCCGGTGGTGAaGGAGAAACCTATTACCGATAAGGAGGTTACTATTCCTACCCCTGTGGCAGAGAAAGAGGAAGTTGCTGCTCCTGTCT

         181      190       200       210       220       230       240       250       260       270
         |---------+---------+---------+---------+---------+---------+---------+---------+---------|
AY045913      CTGATGAGAAGGCGGTTCCAGAGAAGGAGGTGACTCCGGAGAAGGAAGCCCCAGCGGCGGAAGCGGAGAAATCTGTTTCGGTGAAGGAGG
Candidate S38 CTGATGAGAAGGCGGTTCCAGAGAAGGAGGTGACTCCGGAGAAGGAAGCCCCAGCGGCGGAAGCGGAGAAATCTGTTTCGGTGAAGGAGG
Consensus     CTGATGAGAAGGCGGTTCCAGAGAAGGAGGTGACTCCGGAGAAGGAAGCCCCAGCGGCGGAAGCGGAGAAATCTGTTTCGGTGAAGGAGG

         271      280       290       300       310       320       330       340       350       360
         |---------+---------+---------+---------+---------+---------+---------+---------+---------|
AY045913      AAGAGACGGTTGTTGTAGCTGAGAAGGTTGTTGTTTTAACTGCTGAGGAAGTTCAGAAGAAGGCACTTGAGGAGTTTAAGGAGCTTGTAA
Candidate S38 AAGAGACGGTTGTTGTAGCTGAGAAGGTTGTTGTTTTAACTGCTGAGGAAGTTCAGAAGAAGGCACTTGAGGAGTTTAAGGAGCTTGTAA
Consensus     AAGAGACGGTTGTTGTAGCTGAGAAGGTTGTTGTTTTAACTGCTGAGGAAGTTCAGAAGAAGGCACTTGAGGAGTTTAAGGAGCTTGTAA

         361      370       380       390       400       410       420       430       440       450
         |---------+---------+---------+---------+---------+---------+---------+---------+---------|
AY045913      GGGAGGCTTTGAACAAACGTGAATTCACTGCTCCGGTGACGCCGGTTAAGGAAGAGAAAACAGAGGAGAAGAAAACAGAGGAGGAAACTA
Candidate S38 GGGAGGCTTTGAACAAACGTGAATTCACTGCTCCGGTGACGCCGGGGCCTTCGTGGCCTCGAGAGATCTATGAATCGTAGATACTGAAAA
Consensus     GGGAGGCTTTGAACAAACGTGAATTCACTGCTCCGGTGACGCCGGggaaggaagaGaaaacAGAGaacaAgaAAaCagAGaaacaaAaaA

         451      460       470       480       490       500       510       520       530       540
         |---------+---------+---------+---------+---------+---------+---------+---------+---------|
AY045913      AAGAGGAAGAGAAAACAGAGGAGAAGAAAGAAGAGACAACGACTGAGGTTAAGGTTGAAGAAGAGAAACCGGCGGTTCCAGCGGCGGAGG
Candidate S38 ACCCCGCA
Consensus     AacacGaA..................................................................................

         541      550       560       570       580       590       600       610       620       630
         |---------+---------+---------+---------+---------+---------+---------+---------+---------|
AY045913      AGGAGAAATCATCAGAGGCTGCTCCGGTTGAGACCAAATCTGAGGAGAAACCTGAAGAGAAAGCAGAGGTAACAACCGAGAAAGCATCCA
Candidate S38
Consensus     ..................................................................................

         .................................................................................

         1981  1990      2000      2010      20202024
         |---------+---------+---------+---------+---|
AY045913      AAGAACACTTTGTTTCATGATTTCTTATCAAAAAAAAAAAAAAAA
Candidate S38
Consensus     .............................................
```

2. Amino acid sequences of the candidates identified by AtGluRS using yeast two-hybrid system

2.1 Comparison of the amino acid sequences of the candidate S1 to the putative senescence-associated protein ssa-13 [*Pisum sativum*].

```
              1         10        20        30        40        50        60        70        80        90
              |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
SSa-13        TAGARIASSPDSDLEAFSHNPTHGSFAPLAFQPSAMTNCANQRFLSYYVELLLRHCHQWGKTNLSHDGLIPAHVPYWVNNPTLGEFCFT
Candidate S1
Consensus     .........................................................................................

              91        100       110       120       130       140       150       160       170       180
              |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
SSa-13        MIGRADIEGSKSNYAMNAWLPQASYPCGNFSDTSSFKFRSLKDRLATLSRFVFYLEIRIKRAFTLLFHTRFLFSLSSSLGHLRYLLTDVP
Candidate S1     RADIEGSKSNYAMNAWLPQASYPCGNFSDTSSFKFRRSKGSIGHAFTVRIRTENQNQTSFYPFVPHEISVLVELILGHLRYLLTDVP
Consensus     ...RADIEGSKSNYAMNAWLPQASYPCGNFSDTSSFKFRrlKdriahafrfrirlEnrnqraFtllfhhrilflleliLGHLRYLLTDVP

              181       190       200       210       220       230       240       250       260       270
              |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
SSa-13        PQPNSPPDNVFRPDRPTKVSLWIKKEGQRRPPDSRNKLNNVKSSGISTFAVSSSHLSYTSQVISQSRTRVKLNRVFFPADSAKAVPLLLV
Candidate S1  PQPNSPPDNVLRPDRPAEASLGTKNRGCTPPPGLRGLERSMNRRY
Consensus     PQPNSPPDNVlRPDRPaeaSLgiKnrGcrrPPdlRnlernmnrrg........................................

              271       28882
              |---------+-|
SSa-13        SLDSRKGQWEFR
Candidate S1
Consensus     ............
```

2.2 Comparison of the amino acid sequences of the candidate S47 to the putative senescence-associated protein ssa-13 [*Pisum sativum*].

```
              1         10        20        30        40        50        60        70        80        90
              |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
SSa-13        TAGARIASSPDSDLEAFSHNPTHGSFAPLAFQPSAMTNCANQRFLSYYVELLLRHCHQWGKTNLSHDGLIPAHVPYWVNNPTLGEFCFT
Candidate S47
Consensus     .........................................................................................

              91        100       110       120       130       140       150       160       170       180
              |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
SSa-13        MIGRADIEGSKSNYAMNAWLPQASYPCGNFSDTSSFKFRSLKDRLATLSRFVFYLEIRIKRAFTLLFHTRFLFSLSSSLGHLRYLLTDVP
Candidate S47    PDIEGSKSNYAMNAWLPQASYPCGNFSDTSSFKFRRSKGSIGHAFTVRIRTENQNQTSFYPFVPHEISVLVELILGHLRYLLTDVP
Consensus     ....aDIEGSKSNYAMNAWLPQASYPCGNFSDTSSFKFRrlKdriahafrfrirlEnrnqraFtllfhhrilflleliLGHLRYLLTDVP

              181       190       200       210       220       230       240       250       260       270
              |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
SSa-13        PQPNSPPDNVFRPDRPTKVSLWIKKEGQRRPPDSRNKLNNVKSSGISTFAVSSSHLSYTSQVISQSRTRVKLNRVFFPADSAKAVPLLLV
Candidate S47 PQPNSPPDNVLRPDPSWPREIYES
Consensus     PQPNSPPDNVlRPDrptkreiwek........................................

              271       28882
              |---------+-|
SSa-13        SLDSRKGQWEFR
Candidate S47
Consensus     ............
```

2.3 Peptide encoded by candidate S8

R V K L L E S F L E T C T E T S E V V K E S E I R E K V G E K Q S A Q K C L R L
V Q DI Y E S L H L C A V L I V P F P K S L S T Y D T V S E R Q E K L T

2.4 Peptide encoded by candidate S38

Q K D G S R G S T E I G

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